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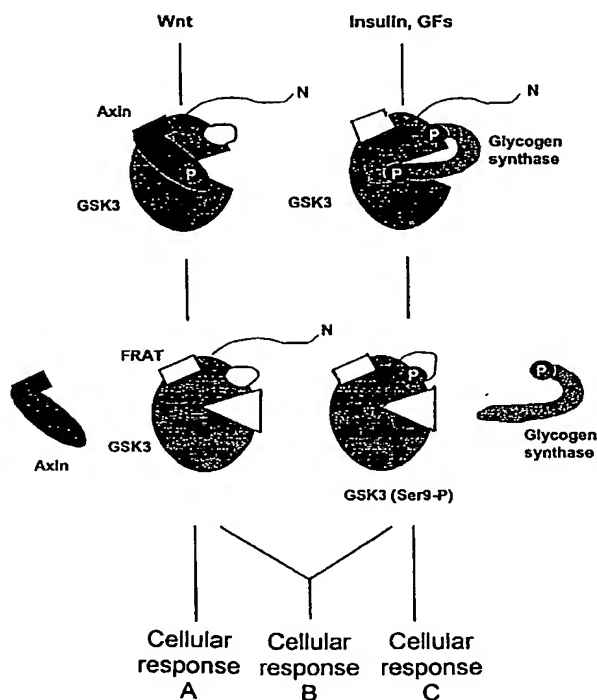
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(54) **Title:** PROTEIN KINASE REGULATION

(57) **Abstract:** Methods are provided for identifying compounds that are capable of, for example, inhibiting the activity of GSK3 towards phosphate-dependent (primed) substrates to a greater extent than towards non-phosphate-dependent substrates. Mutant GSK3s and other novel polypeptides, polynucleotides and recombinant cells that are useful in such methods are provided. For example, polypeptides useful in modulating the activity of GSK3 are also provided.

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PROTEIN KINASE REGULATION

The present invention relates to regulation of protein kinases, particularly GSK3.

5   Glycogen synthase kinase 3 (GSK3) is a protein kinase that has several key functions within cells. Firstly, GSK3 becomes inhibited in response to insulin leading to the dephosphorylation and activation of housekeeping proteins, such as glycogen synthase and eukaryotic protein synthesis initiation factor 2B (eIF2B), and hence to the stimulation of glycogen and

10   protein synthesis ([1] and reviewed in [2]). The inability of insulin to trigger these processes is at the heart of non-insulin dependent or type II diabetes mellitus (NIDDM), the most common disorder of metabolism. Secondly, GSK3 is an essential component of the Wnt signalling pathway, which specifies cell fate during embryonic development (reviewed in [3]).

15   In this pathway GSK3 becomes inhibited in response to Wnts, causing the dephosphorylation of other substrates, including Axin [4] [5] [6], the adenomatous polyposis coli gene product (APC) [7] and β-catenin [8]. Aberrant regulation of this pathway occurs in many human cancers [9]. For example, mutations in APC that disrupt its normal function are commonly

20   found in colorectal cancers, while mutations in β-catenin that make it resistant to degradation are found in many different tumours (reviewed in [10] and [9]). Yet another GSK3 substrate is the microtubule-associated protein tau [11] [12], a hyperphosphorylated form of which is the main component of the neurofibrillary tangles found in the brains of individuals

25   with Alzheimer's disease. These apparently distinct functions of GSK3 highlight the need to control its specificity and regulation very tightly.

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GSK3 is phylogenetically most closely related to the cyclin-dependent protein kinases (CDKs), such as CDK1 (also called cdc2) and CDK2. However, the specificity of GSK3 is unique in that it requires a "primed phosphate" (or "priming phosphate") generally located at n+4 (where n is the site of phosphorylation) in order to phosphorylate many of its substrates, such as glycogen synthase and eIF2B [13]. In contrast, the phosphorylation of Axin and β-catenin in the Wnt signalling pathway is not known to require a "primed" phosphate and appears to rely on high affinity interactions in a multi-protein complex with GSK3 [14] [15] [16]. Thus Axin binds to both GSK3 and β-catenin bringing these proteins into close proximity to facilitate their phosphorylation by GSK3 [6] [17].

GSK3 is fully active in the absence of any signal, but during insulin signal transduction becomes phosphorylated by PKB, in response to agonists that activate phosphatidylinositide 3-kinase, at an N-terminal Ser residue (Ser9 in GSK3β, Ser21 in GSK3α) [18]. It is well established that this phosphorylation inhibits the catalytic activity of the enzyme towards glycogen synthase and eIF2B [1] [2]. However, the mechanism of inhibition is currently not understood.

Inhibition of GSK3 in response to Wnts occurs through a mechanism that is not yet completely understood but which does not involve phosphorylation of the N-terminal serine of GSK3 (Ruel *et al* (1999) *J. Biol. Chem.* 274, 21790-21796: Ding *et al* (2000) *J. Biol. Chem.* 275, 32475-32481).

In this study, we present evidence indicating that there is a specific site of interaction between the phosphate of the "primed" substrate and specific

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residues in GSK3β. In addition, we provide evidence that this same phosphate binding site is also occupied by Ser9 once it becomes phosphorylated by PKB. The existence of this site helps to explain several features of GSK3, such as its unusual substrate specificity and how it becomes inactivated in response to extracellular agonists.

Thus, we identify a phosphate, for example phosphoserine or phosphothreonine, binding site on GSK3 and identify polypeptides that interact with the phosphate binding site. We identify the effect of such polypeptides on the protein kinase activity GSK3 towards different classes of substrate. We identify the mechanism of inactivation of GSK3 by phosphorylation. We also identify a site, alteration of which modulates activity of GSK3 towards non-phosphorylated substrates. We identify assays, mutated polypeptides and substrates that can be used to identify drugs that activate or inhibit the activity of a protein kinase. towards different substrates.

A first aspect of the invention provides a method of identifying a compound that modulates the protein kinase activity of GSK3, comprising the steps of (1) determining the effect of a test compound on the protein kinase activity of GSK3 and/or a mutant thereof, and (2) selecting a compound capable of inhibiting the protein kinase activity of GSK3 towards (i) a phosphate-dependent (primed) substrate and (ii) a non-phosphate dependent (ie non-primed or phosphate-independent, as discussed below) substrate to different extents, wherein when the effect of a test compound is determined on the protein kinase activity of native GSK3 or a fusion thereof but not on a mutant of GSK3, a compound is selected that is capable of inhibiting the protein kinase activity of GSK3 towards a phosphate-dependent (primed)

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substrate to a greater extent than towards a non-phosphate dependent substrate.

It is preferred that the selected compound is capable of inhibiting the protein
5   kinase activity of GSK3 towards a phosphate-dependent (primed) substrate to a greater extent than towards a non-phosphate dependent substrate. However, it may be useful to identify compounds that preferentially inhibit the phosphorylation of non-phosphate dependent substrates when compared with the phosphorylation of phosphate dependent substrates.

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It is particularly preferred that the selected compound is capable of inhibiting the protein kinase activity of active GSK3 towards a phosphate-dependent (primed) substrate to a greater extent than it inhibits the protein kinase activity of active GSK3 towards a non-phosphate dependent
15   substrate. The effect of the test compound may be determined on active or inactivated GSK3 and/or a mutant thereof, as discussed further below.

A further aspect of the invention provides a method of identifying a compound that modulates the protein kinase activity of GSK3, comprising
20· the step of determining the effect of a test compound on the protein kinase activity of inhibited GSK3. The method preferably comprises the step of determining the effect of the compound on the protein kinase activity of inhibited GSK3 towards a non-phosphate dependent (phosphate-independent or non-primed) substrate, for example tau, axin or β-catenin or
25   a dephosphopeptide. Preferably, a compound that increases the protein kinase activity of inhibited GSK3 towards the non-phosphate dependent substrate is selected.

5

The non-phosphate dependent substrate may comprise the sequence S/T-$(X)_n$-Z (wherein n is at least 3, preferably 3, 4, 5 or 6 or up to 14, most preferably 3, and preferably less than about 20, 30 or 40), for example S/T-$(X)_3$-Z and be phosphorylated on the S/T residue by GSK3, wherein Z is not phosphoserine or phosphothreonine (non-primed substrate) and wherein a polypeptide identical to the non-primed substrate with the exception that Z is replaced by a phosphoserine or phosphothreonine residue ("primed" or phosphate-dependent substrate) is a better substrate for phosphorylation on the S/T residue by GSK3 than the non-primed substrate. Z may be serine or threonine, but it is preferred that Z is a non-phosphorylatable residue, and may preferably be alanine.

It is considered that a compound that is capable of activating inhibited GSK3 towards a non-phosphate dependent (phosphate-independent or non-primed) substrate may inhibit the protein kinase activity of GSK3 towards a phosphate-dependent (primed) substrate. A compound that activates inhibited GSK3 towards a polypeptide that does not need to bind to the phosphate binding site (ie releases the inhibition of inhibited GSK3) may be an inhibitor of the phosphorylation of primed substrates, which may need to bind to the phosphate binding site.

Thus, the method may be useful in selecting a compound capable of inhibiting the protein kinase activity of GSK3 (for example active GSK3) towards a phosphate-dependent (primed) substrate to a greater extent than it inhibits the protein kinase activity of GSK3 (for example active GSK3) towards a non-phosphate-dependent (for example phosphate-independent) substrate.

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Inhibited GSK3 may be (1) GSK3 phosphorylated at the residue equivalent to Ser 9 of full-length human GSK3β or (2) GSK3 bound to a compound or polypeptide that interacts with a phosphate binding site of the GSK3 that is defined by residues including arginine 96 of full-length GSK3β, and/or

5   comprising the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, wherein the polypeptide is not a substrate of GSK3. The said compound or polypeptide may also block or restrict access to the catalytic site of GSK3. For example, NT-Ptide-11 may

10   block or restrict access to the catalytic site of GSK3, whereas NT-Ptide-8 is not considered substantially to block or restrict access to the catalytic site of GSK3.

The polypeptide may be a pseudosubstrate of GSK3 ie may be a polypeptide

15   with an amino acid sequence derivable from a phosphate-dependent (primed) substrate of GSK3 in which the serine or threonine residue that is phosphorylatable by GSK3 is replaced by a non-phosphorylatable residue, for example by an alanine residue (or a variant of such a substrate amino acid sequence that retains the ability to bind to GSK3, preferably at the

20   phosphate binding site). For example, the polypeptide may have or comprise a sequence derived from the sequence surrounding the sites in eIF2B or glycogen synthase phosphorylated by GSK3, for example VPPS*PSLS*RHSS*PHQSpEDEEE with one or more of the residues indicated by "S*" changed to a non-phosphorylated/non-phosphorylatable

25   residue, for example alanine. For example, the polypeptide may be a polypeptide   having   the   amino   acid   sequence YRRAAVPPSPSLSRHSAPHQSpEDEEE, where Sp is phosphoserine (pGS in which the serine residue four residues N-terminal to the Sp residue is replaced   by   an   alanine   residue),   or

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PRPASVPPSPSLSRHSAPHQSpEDEEEP   or

PRPASVPPSPSLARHSSpPHQSpEDEEEP. Examples of polypeptides
which may be pseudosubstrate polypeptides are described in Fiol *et al*
(1990) *J Biol Chem* 265(11), 6061-6065. A pseudosubstrate, for example
5   derived from pGS, as indicated above, may have a higher affinity for GSK3
than NT-Ptide (NT-Ptide-11) or NT-Ptide-8. This may mean that less
polypeptide (for example about twenty times less than if NT-Ptide (NT-
Ptide-11 or NT-Ptide-8) were used) may be required when performing a
screening assay in order to provide inhibited GSK3. A pseudosubstrate
10   polypeptide may not comprise an amino acid sequence corresponding to the
motif S/T-X-X-X-Sp/Tp or S/T-$(X)_n$-Sp/Tp (wherein n is at least 3,
preferably 3, 4, 5 or 6 or up to 14, most preferably 3, and preferably less
than about 20, 30 or 40). It may comprise an amino acid sequence
corresponding to the motif Z1-X-X-X-Sp/Tp, wherein Z1 is a non-
15   phosphorylatable residue, for example alanine.   Z1 is not serine or
threonine. It may be preferred that the polypeptide comprises one or more
proline residues, but the presence or position of such a residue does not
appear to be critical.   For example one or more of the residues
corresponding to an X residue in the above motif may be a proline residue.

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Thus, pseudosubstrates derivable from known primed substrates could be
used as an alternative to, for example, NT-Ptide (NT-Ptide-11) to block the
total activity of GSK3 in search of compounds that may activate GSK3
against non-phosphate dependent (for example non-primed) substrates and
25   inhibit phosphorylation of primed substrates.

The protein kinase activity of GSK3 phosphorylated at the residue
equivalent to Ser9 of full length GSK3β is inhibited when compared with
that of Ser9 (or equivalent residue) unphosphorylated (active) GSK3.

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Although not intending to be bound by theory, we consider, as discussed in Example 1, that this inhibition may arise by binding of the phosphorylated N-terminus of GSK3 to the phosphate binding site as a "pseudosubstrate". A compound which can displace the phosphorylated N-terminus from the

5   phosphate binding site may activate GSK3 towards non-phosphate-dependent (for example non-primed) substrates but not towards primed substrates.

Active GSK3 may be phosphorylated (probably on tyrosine), but not on the

10   residue equivalent to Ser9 of full length human GSK3β.

Thus, the invention provides a method of identifying a compound that modulates the protein kinase activity of GSK3, wherein the effect of the said compound on the rate or degree (including position) of phosphorylation of a

15   substrate polypeptide of GSK3 by GSK3 in the presence of an interacting compound or polypeptide is determined, and a compound that modulates the said rate or degree of phosphorylation is selected, wherein the interacting compound or polypeptide interacts with the phosphate binding site of the GSK3 that is defined by residues including arginine 96 of full-length

20   GSK3β and/or comprises the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, wherein the polypeptide is not a substrate of GSK3.   The polypeptide may be a pseudosubstrate of GSK3, as discussed above.

25

A still further aspect of the invention provides a method of identifying a compound that modulates the protein kinase activity of GSK3, comprising the step of determining the effect of the compound on the phosphorylation by GSK3 of a non-primed polypeptide, wherein the non-primed polypeptide

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comprises the sequence S/T-$(X)_n$-Z (wherein n is at least 3, preferably 3, 4, 5 or 6 or up to 14, most preferably 3, and preferably less than about 20, 30 or 40) and is phosphorylated on the S/T residue by GSK3, wherein Z is not phosphoserine or phosphothreonine and wherein a polypeptide identical to the non-primed polypeptide with the exception that Z is replaced by a phosphoserine or phosphothreonine residue (primed substrate) is a better substrate for phosphorylation on the S/T residue by GSK3 than the non-primed substrate. The Z residue may in theory be any linear distance away from the S/T residue (ie an upper limit for n may not be appropriate). Priming is more dependent on the tertiary structure to bring the primed residue into close spatial proximity to the residue to be phosphorylated. The primed substrate may preferably be a naturally occurring primed substrate of GSK3 or a fragment or fusion thereof.

Measuring the effect of a compound on phosphorylation of such a non-primed polypeptide and preferably also of the equivalent primed polypeptide by GSK3, may allow compounds which interact with the phosphate binding site to be identified. A compound which inhibits only the phosphorylation of the primed polypeptide may bind to the phosphate binding site of GSK3.

The GSK3 may be active or inhibited. The assay may preferably be performed using phosphorylated GSK3 or in the presence of an interacting polypeptide or compound, as discussed in relation to previous aspects of the invention.

A further aspect of the invention provides a method of identifying a compound that modulates the protein kinase activity of GSK3, comprising the step of determining the effect of the compound on the protein kinase

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activity of, or ability of the compound to bind to, (1) GSK3 mutated at a residue defining at least part of the phosphate binding site of GSK3 that is defined by residues including arginine 96 of full-length GSK3β, for example the residue equivalent to arginine 96, and/or the residue equivalent

5   to lysine 94, of full-length human GSK3β and/or (2) GSK3 mutated at a residue defining at least part of the GSK3 site, alteration of which modulates activity of GSK3 towards non-phosphorylated substrates, that is defined by residues including leucine 128 of full-length human GSK3β, for example the residue equivalent to leucine 128 of full-length human GSK3β.

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Mutation of the residue equivalent to arginine 96 of full-length human GSK3β (for example to Ala or Lys) is considered to disrupt the phosphate binding site of GSK3. Mutation of the residue equivalent to lysine 94 of full-length human GSK3β, particularly to glutamate is considered to affect

15   phosphorylation of primed substrates. Mutation of this residue to alanine has less effect. Mutation of this residue, for example to Glu, may affect the site, but the residue may not be an essential part in the phosphate binding site, ie may not be directly involved in the interaction with the primed phosphate.

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Mutation of the residue equivalent to leucine 128 of full-length human GSK3β is considered to affect the phosphorylation of axin (which does not require priming phosphate). It is preferred that the relevant residue is mutated to an alanine residue.

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Preferably the method further comprises the step of determining the effect of the compound on the protein kinase activity of, or ability of the compound to bind to, GSK3 which is not mutated at the residue (1) defining at least part of the phosphate binding site of GSK3 that is defined by

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residues including arginine 96 of full-length GSK3β, for example the residue equivalent to arginine 96, and/or the residue equivalent to lysine 94, of full-length human GSK3β and/or (2) defining at least part of the GSK3 site, alteration of which modulates activity of GSK3 towards non-

5   phosphorylated substrates, that is defined by residues including leucine 128 of full-length human GSK3β, for example the residue equivalent to leucine 128 of full-length human GSK3β. Preferably, the GSK3 is active, for example not phosphorylated on the residue equivalent to Ser9 of full-length human GSK3β.

10

As discussed in Example 1, a compound may have a different effect on the mutated and non-mutated GSK3. Thus, a compound which interacts with the phosphate binding site may modulate the protein kinase activity of wild-type GSK3 and GSK3 mutated at the residue equivalent to leucine 128 but

15   may not affect the protein kinase activity of GSK3 mutated at the residue equivalent to arginine 96.

By primed substrate is meant a polypeptide which comprises the sequence S/T-$(X)_n$-Z (wherein n is at least 3, preferably 3, 4, 5 or 6, or up to 14, most

20   preferably 3, and preferably less than about 20, 30 or 40, as discussed above) and is phosphorylated on the S/T residue by GSK3, wherein Z is phosphoserine (Sp) or phosphothreonine (Tp). Examples of primed substrates include Glycogen synthase, eIF2B and fragments thereof, for example pGS and the eIF2B fragment as discussed in Examples 1 and 2.

25

By non-primed substrate is meant a substrate of GSK3 that is identical to a primed substrate except that the phosphoserine or phosphothreonine residue is replaced with a different residue, for example alanine. Such a substrate may be phosphorylated at a much lower rate by GSK3 than a primed or

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phosphate-independent substrate, as discussed in Example 1. The extent of phosphorylation of a substrate by GSK3 should be initially linearly related to the amount of enzyme used and time of incubation.

5   By phosphate-independent substrate is meant a substrate which does not require the presence of a primed phosphorylation in order for phosphorylation by GSK3 to occur at a particular serine or threonine residue. These substrates seem to bind to GSK3 through sites other than the phosphate binding site. Examples of phosphate-independent substrates
10   include Axin, β-catenin, Tau, and probably c-myc, myb and c-jun.

It will be appreciated that a polypeptide may be both a primed and a phosphate-independent substrate. Thus, a polypeptide may be phosphorylated on one or more residues in a phosphate-dependent manner,
15   and on one or more other residues in a phosphate-independent manner. Further, a given residue may be phosphorylated by GSK3 in either a phosphate-dependent or phosphate-independent manner, possibly with different binding affinity for GSK3 when the substrate is already phosphorylated at a priming residue to when it is not so phosphorylated
20   (primed).

Thus, non primed substrates, for example axin, may have a strong interaction with GSK3 at a site distinct from the active site and the phosphate binding site and these substrates can be phosphorylated in the
25   absence of a primed site at position n+4. Sites in axin that are phosphorylated have been mapped *in vitro* using various isoforms of axin. Different groups have identified different sites, suggesting that there are likely to multiple phosphorylation sites *in vitro*. Ruel *et al* (1999) *J Biol Chem* **274(31)**, 21790-21796, for example, defines the smallest region of

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axin phosphorylated by GSK3 as between residues 356 and 383. There are nine Ser/Thr residues within this region, four of which lie in S-X-X-X-S motifs, though axin is not considered to require prior priming in order to be phosphorylated by GSK3. Ikeda *et al* (1998) *EMBO J* 17(5), 1371-1384

5   reached a similar conclusion using rat axin. The region identified (298-506) spans the region defined by Ruel *et al*. Jho *et al* (1999) *Biochem Biophys Res Commun* 266, 28-35 claims that only the region 600-672 is efficiently phosphorylated *in vitro*, and that multiple sites are phosphorylated *in vivo*. T609 and S614 are indicated to be physiological GSK3β target sites.

10   However, these studies were performed with S/A mutants only and were not confirmed by mass spectroscopy or cycle burst analysis, so the conclusions may not be correct; incorrect conclusions have previously been drawn from studies using mutants. It is not clear which sites are phosphorylated in cells. Most of the sites identified *in vitro* have a serine a position $n+4$. Axin may

15   also be phosphorylated at a serine residue located at the $n+4$ position, and therefore in addition it may interact with the phosphate binding site of GSK3. In this case, a compound directed to the phosphate binding site may inhibit phosphorylation of other primed substrates - which may lack other strong interaction sites with GSK3 - to a greater extent than they would

20   inhibit axin. This is because a compound directed to the phosphate binding site may not greatly affect axin interaction with GSK3. Therefore even if axin can be a primed substrate as well as a non-primed substrate, it could still be less affected by a compound directed against the phosphate binding site, than other primed substrates.

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The classification of GSK3 substrates as "primed" or "non-primed" may be a simplification. Substrates may have several different interaction sites with GSK3 and different substrates may depend on one binding site more than others.

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It may be preferred that a compound identified or designed using a method of the invention is capable of modulating, preferably inhibiting the phosphorylation by GSK3 (preferably active GSK3) of a first substrate to a

5   different extent to that of a second (or further) substrate. The first and second substrates may both be phosphate-dependent or phosphate independent substrates. As will be clear from the discussion above, the first and second substrates may be different phosphorylation sites on the same polypeptide, or even the same phosphorylation site in the presence and

10   absence of a priming phosphorylated residue.

The effect of the compound on the rate or degree of phosphorylation of a phosphate-dependent ("primed") substrate, for example glycogen synthase or eIF2B or suitable variant, fragment, derivative or fusion thereof, may be

15   determined.

The effect of the compound on the rate or degree of phosphorylation of a non-phosphate-dependent substrate, for example axin or β-catenin or suitable variant, fragment, derivative or fusion thereof, may be determined.

20   Suitable fragments of axin include residues 280 to 500, or 275 to 510 of human axin.

A further aspect of the invention provides a method of identifying a compound that modulates the protein kinase activity of GSK3, wherein the

25   ability of the compound to inhibit, promote or mimic the interaction of GSK3 with an interacting compound or polypeptide is measured and a compound that inhibits, promotes or mimics the said interaction is selected, wherein the interacting compound or polypeptide interacts with the phosphate binding site of the GSK3 that is defined by residues including

15

arginine 96 of full-length GSK3β and/or comprises the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, wherein the polypeptide is not a substrate of GSK3.

In this and previous aspects of the invention, the interacting polypeptide may be part of the GSK3 polypeptide chain. Intramolecular interactions may be detected using, for example, FRET techniques, as described further in Example 3. Methods of measuring the interaction and properties of the GSK3 and interacting polypeptide or compound are discussed further below.

A method of the invention preferably further comprises the step of selecting a compound that decreases the protein kinase activity of GSK3 (preferably active GSK3) towards a primed substrate and does not affect or increases the protein kinase activity of GSK3 towards a non-phosphate-dependent substrate.

The term GSK3 as used herein includes a polypeptide (a GSK3 polypeptide) comprising the amino acid sequence identified as GSK3 in NCBI accession number NM-002093, Stambolic and Woodgett (1994) *Biochemical Journal* 303, 701-704, or a variant, fragment, fusion or derivative thereof, or a fusion of a said variant or fragment or derivative. It is preferred that the said GSK3 polypeptide is a protein kinase. It is preferred that the said GSK3 polypeptide is a protein kinase that is capable of phosphorylating a serine or threonine residue that lies in a S/T-(X)$_n$-Sp/Tp (wherein n is at least 3, preferably 3, 4, 5 or 6 or up to 14, most preferably 3, and preferably less than about 20, 30 or 40, as discussed above) consensus motif (where the underlined S/T corresponds to the serine or threonine that is phosphorylated by GSK3 and X is a variable residue), and preferably that is capable of

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phosphorylating glycogen synthase or pGS. The rate at which the said GSK3 polypeptide is capable of phosphorylating a serine or threonine residue as described above may be increased by dephosphorylation of GSK3 by phosphatase PP2a. It is preferred that the said GSK3 polypeptide is also

5   capable of phosphorylating Axin, β-catenin or Tau. It may further be preferred that the substrate specificity and/or other characteristics of the said GSK3 polypeptide *in vitro* may be substantially as reported in Methods in Enzymology (1991) **200**, 565-577.

10   The following EMBL database records relate to the polypeptides indicated:

NM_002093 Homo sapiens glycogen synthase kinase 3 beta (GSK3B) mRNA. VERSION   NM_002093.1 GI:4504162

NM_004655 Homo sapiens axin 2 (conductin, axil) (AXIN2), mRNA.
15   VERSION   NM_004655.1 GI:4757823

D63424 Homo sapiens mRNA for glycogen synthase kinase 3alpha, complete cds. Adult brain. VERSION   D63424.1 GI:2641993

20   UGS1_HUMAN GLYCOGEN [STARCH] SYNTHASE, MUSCLE.
ACCESSION P13807; PID   g1351366
VERSION   P13807 GI:1351366

A38973 beta-catenin - human.
25   ACCESSION A38973; PID   g2134817
VERSION   A38973 GI:2134817

AAC51624 axin [Homo sapiens].
ACCESSION AAC51624; PID   g2252820
30   VERSION   AAC51624.1 GI:2252820
DBSOURCE   locus AF009674 accession AF009674.1

Tau – there are multiple isoforms of tau generated by alternative splicing. The following record relates to the full-length protein isoform 1.

35

NP_058519 microtubule-associated protein tau, isoform 1 [Homo sapiens].
ACCESSION NP_058519; PID   g8400713

17
VERSION   NP_058519.1 GI:8400713
DBSOURCE   REFSEQ: accession NM_016835.1

5   eIF2B – the epsilon isoform record is given here since this was the one
     originally shown to be phosphorylated by GSK3.

     AAC50646 eIF-2Bepsilon.
     ACCESSION   AAC50646; PID   g806854
10   VERSION   AAC50646.1 GI:806854
     DBSOURCE   locus HSU23028 accession U23028.1

     FRT1_HUMAN   PROTO-ONCOGENE   FRAT1   (FREQUENTLY
     REARRANGED IN ADVANCED T-CELL LYMPHOMAS).
15   ACCESSION   Q92837; PID   g6226616
     VERSION   Q92837 GI:6226616
     DBSOURCE   swissprot: locus FRT1_HUMAN, accession Q92837;

20   FRAT2 and FRAT3 share some homology with FRAT1 and are likely to
     share some functions of FRAT1; they may be used in assays in place of
     FRAT1. Axil/Conductin are considered to be very similar to Axin and may
     be used in assays in place of Axin.

25   It is particularly preferred, although not essential, that the variant or
     fragment or derivative or fusion of the GSK3, or the fusion of the variant or
     fragment or derivative has at least 30% of the enzyme activity of full-length
     human GSK3β with respect to the phosphorylation of pGS or GS (see
     Example 1) when the GSK3 is activated (ie not phosphorylated). It is more
30   preferred if the variant or fragment or derivative or fusion of the said GSK3,
     or the fusion of the variant or fragment or derivative has at least 50%,
     preferably at least 70% and more preferably at least 90% of the enzyme
     activity of GSK3β with respect to the phosphorylation of pGS or GS.
     However, it will be appreciated that variants or fusions or derivatives or
35   fragments which are devoid of enzymatic activity may nevertheless be

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useful, for example by interacting with another polypeptide. Thus, variants or fusions or derivatives or fragments which are devoid of enzymatic activity may be useful in a binding assay, which may be used, for example, in a method of the invention in which modulation of an interaction of GSK3

5   (as defined above) with a interacting polypeptide, for example an interacting polypeptide comprising the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, is measured.

10   As described in Example 1 a R96A GSK3 mutant has less then 1% of the activity of the full-length wild-type protein towards pGS, but has similar activity to the wild-type enzyme towards GS. Such a mutant may be very useful, for example in screening assays, as indicated above. Thus, it will be appreciated that it is not necessary for the variants or fusions or derivatives

15   or fragments to retain a stated level of activity against both pGS and GS.

It is preferred that the variant or fragment or derivative or fusion of the said GSK3, or the fusion of the variant or fragment or derivative comprises a phosphate binding site that is defined by residues including arginine 96 of

20   full-length human GSK3β, as discussed further below.

By "variants" of a polypeptide we include insertions, deletions and substitutions, either conservative or non-conservative. In particular we include variants of the polypeptide where such changes do not substantially

25   alter the activity of GSK3, as described above.

By "conservative substitutions" is intended combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

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It is particularly preferred if the GSK3 variant has an amino acid sequence which has at least 65% identity with the amino acid sequence of GSK3β or GSK3α referred to above, more preferably at least 70%, 71%, 72%, 73% or 74%, still more preferably at least 75%, yet still more preferably at least

5   80%, in further preference at least 85%, in still further preference at least 90% and most preferably at least 95% or 97% identity with the amino acid sequence defined above.

It is still further preferred if the GSK3 variant has an amino acid sequence

10   which has at least 65% identity with the amino acid sequence of the catalytic domain, particularly the residues forming the phosphate binding site, of GSK3 in the appropriate sequence referred to above, more preferably at least 70%, 71%, 72%, 73% or 74%, still more preferably at least 75%, yet still more preferably at least 80%, in further preference at least 83 or 85%,

15   in still further preference at least 90% and most preferably at least 95% or 97% identity with the amino acid sequence defined above. It will be appreciated that the catalytic domain of a protein kinase-related polypeptide may be readily identified by a person skilled in the art, for example using sequence comparisons as described below.

20

The percent sequence identity between two polypeptides may be determined using suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group and it will be appreciated that percent identity is calculated in relation to polypeptides

25   whose sequence has been aligned optimally.

The alignment may alternatively be carried out using the Clustal W program (Thompson *et al* (1994) *Nucl Acid Res* 22, 4673-4680). The parameters used may be as follows:

Fast pairwise alignment parameters: K-tuple(word) size; 1, window size; 5, gap penalty; 3, number of top diagonals; 5. Scoring method: x percent. Multiple alignment parameters: gap open penalty; 10, gap extension penalty; 0.05.

5   Scoring matrix: BLOSUM.

It is preferred that the GSK3 is a polypeptide which consists of the amino acid sequence of the GSK3 sequence referred to above or naturally occurring allelic variants thereof. It is preferred that the naturally occurring

10   allelic variants are mammalian, preferably human, but may alternatively be homologues from parasitic or pathogenic or potentially pathogenic organisms.

A *S pombe* homologue of GSK3 may be Skp1+, described in Plyte *et al*

15   (1997) *Mol Cell Biol* **17(3)**, 1756 and references therein.

The GSK3 may be Myc epitope-tagged or His-tagged or GST-tagged, as described in Example 1. It may be a GFP (green fluorescent protein) fusion, as described in Example 2 and known to those skilled in the art.

20

It is preferred that the GSK3 is a polypeptide that is capable of interacting with a polypeptide comprising the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof or

25   pseudosubstrate such as that based on pGS, as described above. Further preferences for the said polypeptide are as given above in relation to the interacting polypeptide.

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The capability of the said GSK3 polypeptide with regard to interacting with or binding to a polypeptide, for example a polypeptide comprising the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8)

5   or variant thereof, may be measured by any method of detecting/measuring a protein/protein interaction, as discussed further below. Suitable methods include methods analogous to those discussed above and described in Example 1 or Example 2, for example yeast two-hybrid interactions, co-purification, ELISA, co-immunoprecipitation, scintillation proximity assay

10 (SPA) and surface plasmon resonance methods. Phage display techniques may be used, as known to those skilled in the art and discussed further in Example 2. It is particularly preferred that a fluorescence resonance energy transfer (FRET) method is used, as described in Example 2. Thus, the said GSK3 may be considered capable of binding to or interacting with a

15 polypeptide, for example a polypeptide comprising the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof (or a pseudosubstrate as discussed above), if an interaction may be detected between the said GSK3 polypeptide and the said interacting polypeptide by

20 ELISA, co-immunoprecipitation, scintillation proximity assay (SPA) or surface plasmon resonance methods (for example methods analogous to those described in Balendran *et al* (1999) *Curr. Biol.* 9, 393-404; UK patent application No 9906245.7 filed 19 March 1999 and Alessi *et al* US patent application filed 2 December 1999, or by a yeast two-hybrid interaction or

25 copurification method or a FRET method, for example as described in Example 1.

It is preferred that the interaction can be detected using a surface plasmon resonance method, as described in Balendran *et al* (1999) *supra*; UK patent

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application No 9906245.7 *supra* and Alessi *et al* US patent application *supra*. The interacting polypeptide may be immobilised on the test surface, for example it can be coupled through amino groups to a SensorChip CM5™, according to the manufacturer's instructions, or a biotinylated

5   polypeptide can be bound to an avidin coated SensorChip SA. The protein kinase (at concentrations between, for example 0 and between 10μM and 1.0μM, for example 2μM) is then injected over the surface and steady state binding determined in each case. From these measurements a K_d can be determined. It is preferred that the interaction has a K_d of less than 50 μM,

10   20 μM or 8μM, more preferably less than 5μM, 2μM, 1μM, 800nM, 500nM, 300nM, 200nM or 100nM, for example about 500nM. Alternatively, a K_d can be determined for a polypeptide in competition with the immobilised polypeptide. The protein kinase (for example at a concentration of 0.5μM) is mixed with free polypeptide (for example, at concentrations between 0 and

15   3μM) and the mixture injected over the immobilised polypeptides. The steady state binding is determined in each case, from which the K_d of the interaction can be determined using the Cheng-Prescott relationship. Alternatively, the interaction may be expressed in terms of an observed response or relative observed responses, measured in terms of mass of

20   protein bound to the surface, as described in Balendran *et al* (1999) *supra* and the cited patent applications. For example, the polypeptide may be immobilised by amino coupling to a SensorChip CM5 and each protein kinase (for example different mutated protein kinases, as discussed below) for example at a concentration of 1.0μM, injected over the immobilised

25   polypeptide. Alternatively, the polypeptide may be immobilised on a SA SensorChip and each protein kinase, for example at a concentration of 40nM injected over the immobilised polypeptide. The steady state response for each protein kinase is determined, for example expressed in Response Units (RU). 1000RU corresponds to 1 ng/mm^2 of protein bound to the surface. A

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response of less than 10RU may indicate that no interaction has taken place. A response of at least 10RU may indicate that the immobilised and injected molecules interact with each other.

5   It will be appreciated that the above methods may be used to determine whether a particular polypeptide is an interacting polypeptide in respect of GSK3.

An amino acid sequence may be identifiable as that of a protein kinase
10   catalytic domain by reference to sequence identity or similarities of three dimensional structure with known protein kinase domains, as known to those skilled in the art.

Protein kinases show a conserved catalytic core, as reviewed in Johnson *et*
15   *al* (1996) *Cell*, 85, 149-158 and Taylor & Radzio-Andzelm (1994) *Structure* 2, 345-355. This core folds into a small N-terminal lobe largely comprising anti-parallel β-sheet, and a large C-terminal lobe which is mostly α-helical. A deep cleft at the interface between these lobes is the site of ATP binding, with the phosphate groups near the opening of the cleft.

20

Protein kinases also show conserved sequences within this catalytic core, and the residue equivalent to a given residue of, for example, GSK3, may be identified by alignment of the sequence of the kinase with that of known kinases in such a way as to maximise the match between the sequences.
25   The alignment may be carried out by visual inspection and/or by the use of suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group, which will also allow the percent identity of the polypeptides to be calculated. The Align program (Pearson (1994) in: Methods in Molecular Biology, Computer Analysis of

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Sequence Data, Part II (Griffin, AM and Griffin, HG eds) pp 365-389, Humana Press, Clifton).

The comparison of amino acid sequences or three dimension structure (for example from crystallography or computer modelling based on a known structure) may be carried out using methods well known to the skilled man, as detailed below and as described in Example 1.

The interacting polypeptide or compound may interact with the said phosphate binding site of GKS3. Thus, it is preferred that the interacting polypeptide interacts with GSK3 but interacts less strongly with GSK3 in which one or more residues forming the said phosphate binding site is mutated, preferably to a non-conserved amino acid. Most preferably, the mutated residue is the residue equivalent to residue Arg 96 of full length GSK3β. It is particularly preferred that the residue at the position equivalent to residue Arg 96 of GSK3β is mutated to an Ala. It may alternatively be mutated to another type of residue, including the basic residue Lys; as described in Example 1, mutation to Lys is sufficient to disrupt the phosphate binding site.

It will be appreciated that the interacting polypeptide or compound may interact with additional regions of the protein kinase, for example other portions of the active site. A compound that interacts with the phosphate binding site but does not block the catalytic site of GSK3 may be particularly useful. Such a compound may be identified, for example, by kinetic and competition experiments, as will be apparent to those skilled in the art. It is considered that such a compound may inhibit phosphorylation of primed substrates without inhibiting phosphorylation of non-phosphate dependent substrates such as Axin and β-catenin.

The interaction may be measured by any of the methods discussed above. In particular, it may be measured using scintillation proximity assay techniques or surface plasmon resonance, as discussed above and in Balendran *et al* (1999) *supra*. It is particularly preferred that the relative strength of interaction with the GSK3 and the mutated GSK3 is determined by measuring the relative steady state responses, as described above. It is preferred that the response (expressed in RUs) for the unmutated protein kinase is at least 2, 5, 10, 30, 50, 80, 100, 200 or 500 times the response for the mutated protein kinase.

The interacting polypeptide, for example comprising the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, may be part of the same polypeptide chain as the GSK3. For example, phosphorylated GSK3 comprises the amino acid sequence RPRTTSpFAESC (GSK3β) or RARTSSpFAEPG (GSK3α); consensus for these two = R P/A RT T/S SpFAE S/P C/G (or a shorter portion thereof, for example T T/S SpFAE S/P C/G). Thus, the interaction may be an intramolecular interaction, for example in which the phosphate binding site (of the protein kinase domain of the polypeptide) and the interacting portion of the polypeptide, for example a portion of the polypeptide comprising a A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E sequence, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, within a single polypeptide chain, interact. Alternatively, but less probable, two or more such polypeptide chains may form a dimer or multimer through intermolecular interactions between, for example, the phosphate binding site of one polypeptide chain and the interacting portion of a second polypeptide. Intramolecular interactions can be measured by

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techniques known to those skilled in the art, including cross-linking studies, structural studies and fluorescence resonance energy transfer (FRET) methods, in which changes in separation between fluorophores, for example attached to different parts of a molecule, can be measured. This is discussed

5   in more detail in Example 2.

A polypeptide comprising the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, or pseudosubstrate

10   sequence, may interact with a said phosphate binding site of GSK3 with different affinity depending upon the phosphorylation state of the Ser/Thr residue. Thus, the polypeptide may interact with the phosphate binding site more strongly when phosphorylated on the Ser/Thr residue than when not so phosphorylated. In the absence of phosphorylation, the interaction may be

15   substantially undetectable using one or more of the methods described above or may be about 2, 5 or 10-fold weaker than when phosphorylated. Thus, for example, an intra- or intermolecular interaction between the GSK3 protein kinase domain and the portion comprising the sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-

20   Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, may occur substantially only when the said sequence is phosphorylated on the Ser/Thr residue. The interaction may modulate, for example decrease, the activity and/or stability of the GSK3. The interaction may increase stability of the GSK3.

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It is preferred that the interacting polypeptide, for example comprising the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, is a polypeptide that is capable of binding

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GSK3 and inhibiting its activity towards pGS in substantially the same way as a polypeptide with the amino acid sequence RPRTTSpFAESC (NT-Ptide), or in substantially the same way as a polypeptide with the amino acid sequence TTSpFAESC (NT-Ptide-8), as described in Example 1.

5

The one letter or three-letter amino acid code of the IUPAC-IUB Biochemical Nomenclature Commission are used herein, with the exception of the symbols Z and Z1, defined above. In particular, X represents any amino acid. It is preferred that X, Z and Z1 represent a naturally occuring

10   amino acid. It is preferred that at least the amino acids corresponding to the consensus sequences defined above are L-amino acids.

By modulation of the protein kinase activity is included inhibition or an increase in the protein kinase activity. Modulation includes a change in the

15   pattern of relative phosphorylation of different substrates. An increase of activity of a non-inhibited form of GSK3 may arise, for example through induction of conformational change.

The protein kinase activity of GSK3 that is modulated may be

20   phosphorylation of the underlined residue in a polypeptide with the amino acid sequence $\underline{S/T}$-$(X)_n$-Sp/Tp (wherein n is at least 3, preferably 3, 4, 5 or 6, most preferably 3). Alternatively or in addition, the modulated activity may be phosphorylation of a non-phosphate dependent substrate, for example GS, axin, β-catenin or Tau. No consensus sequence has been

25   determined for non-phosphate dependent substrates. However, sites in c-myb and c-myc may be clustered and in proline-rich acidic regions. Thus, non-phosphate dependent phosphorylation sites may be found in proline-rich acidic regions.

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A compound identified by a method of the invention may modulate the ability of the protein kinase to phosphorylate different substrates, for example different naturally occurring polypeptides, to different extents. The compound may inhibit the protein kinase activity in relation to one substrate

5   but may increase or not affect the protein kinase activity in relation to a second substrate, for example as discussed in Example 1. A compound at a given concentration may inhibit phosphorylation of one substrate to a greater extent than another substrate. Thus, a compound may have different IC_{50}s in relation to phosphorylation of different substrates. The term IC_{50}

10   is well known to those skilled in the art and indicates the concentration of compound necessary to inhibit the observed parameter (ie phosphorylation of a particular substrate under particular conditions) to 50% of the value in the absence of the compound. The lower the IC_{50}, the more potent the compound. Methods of calculating IC_{50} values are well known to those

15   skilled in the art. A similar measure of the effect of the compound may be calculated for compounds that increase phosphorylation of a given substrate (for example the concentration necessary to increase phosphorylation by, for example 20% or 50%).

20   NT-Ptide-8 inhibits phosphorylation of primed substrates, but not of non-primed substrates, for example Axin, as described in Example 1.

It will be appreciated that the modulatory, for example inhibitory action of a compound found to bind (or inhibit binding of a polypeptide or compound)

25   to the protein kinase may be confirmed by performing an assay of enzymic activity (for example with respect to primed, non-primed or phosphate-independent substrates) in the presence of the compound.

The said interacting polypeptide may be derivable from GSK3.

Thus, the interacting polypeptide may comprise or consist essentially of the amino acid sequence from residues 4, 5, 6 or 7 (preferably 4 or 7) to 14 of the N-terminus of GSK3β or from residues 16, 17, 18 or 19 (preferably 16
5   or 19) to 26 of GSK3α.

The said interacting polypeptide may comprise or consist essentially of the sequence RPRTTSpFAESC or TTSpFAESC (derivable from GSK3β) or TSSpFAEPG or RARTSSpFAEPG (derivable from GSK3α).

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The said interacting polypeptide may be derivable from a primed substrate of GSK3 in which the serine or threonine residue(s) that is phosphorylated by GSK3 is replaced by a non-phosphorylatable residue, for example alanine. Thus, the said interacting polypeptide may be derivable from
15   glycogen synthase, particularly from the peptide pGS, with such replacement (s), as discussed above.

The said interacting polypeptide may comprise or consist essentially of a variant of a glycogen synthase or GSK3 sequence indicated above.
20   Preferably, in such a variant of a GSK3 sequence, the residues that correspond to the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E in the sequence indicated above are unchanged, or, if changed, still have the sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E. It is preferred that the residues within about 2, 5 or 10 amino acids C- or N-
25   terminal of the phosphoserine residue or non-phosphorylatable residue (at the site where a serine or threonine residue would be phosphorylated) are also unchanged.

30

It is preferred that the interacting polypeptide has fewer than about 400, 380, 350, 300, 250, 200, 150, 100, 80, 50, 40 or 30 amino acids.

5   The said interacting polypeptide may comprise a GST portion, as described in Example 1. This may be useful in purifying and/or detecting the said interacting polypeptide. The said interacting polypeptide may be biotinylated or otherwise tagged, for example with a 6His, HA, myc or other epitope tag, as known to those skilled in the art. The interacting polypeptide may additionally or alternatively comprise a chromogenic portion, for

10   example a fluorophore, for example a GFP portion, as described in Example 2.

The effect of the compound may be determined by comparing the rate or degree of phosphorylation of the said substrate polypeptide by the said

15   GSK3 or mutant thereof in the presence of different concentrations of the compound, for example in the absence and in the presence of the compound, for example at a concentration of about 100μM, 30μM, 10μM, 3μM, 1μM, 0.1μM, 0.01μM and/or 0.001μM.

20   It will be appreciated that the compound may interact with GSK3 or with the said interacting polypeptide or with both.

The compound may mimic the effect of the interaction of an interacting polypeptide (that interacts with the phosphate binding site of the protein

25   kinase and/or comprises the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof) with GSK3.

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A compound that mimics the effect of an interacting polypeptide on GSK3 may decrease the rate or extent of phosphorylation of a primed, non-primed or non-phosphate dependent substrate polypeptide by GSK3. The extent of the decrease may be different for different substrates.

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By "mimics the effect" of the interaction of the said interacting polypeptide with the GSK3 is meant that the compound has a quantitative or qualitative effect on the GSK3, for example on its protein kinase activity, that is the same as an effect of the interacting polypeptide on the protein kinase, for

10   example on its protein kinase activity, as discussed in Example 1.

The GSK3 and interacting polypeptide may form a complex, which may be detected in a cell-free system, for example by BiaCore measurements. The ability of the compound to inhibit or promote the formation or stability of

15   the complex may be determined by exposing the protein kinase and/or interacting polypeptide and/or complex of the protein kinase and interacting polypeptide to the compound and determining any change in the affinity, extent or stability of the interaction in the presence of the compound.

20   It is preferred that the said GSK3, interacting polypeptide and/or, where appropriate, substrate polypeptide, is a recombinant or synthetic polypeptide. It is further preferred that the said GSK3, interacting polypeptide and/or, where appropriate, substrate polypeptide is substantially pure when introduced into the method of the invention.

25

By "substantially pure" we mean that the GSK3 or interacting polypeptide or substrate polypeptide is substantially free of other protein kinases, phosphatases and peptidases, and preferably other proteins. Thus, we include any composition that includes at least 5%, 10%, 20% or 30% of the

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protein content by weight as the said GSK3 or interacting polypeptide or substrate polypeptide, preferably at least 20%, 30%, 40% or 50%, (or possibly more preferably at least 70%, still more preferably at least 90% and most preferably at least 95%) of the protein content is the said protein kinase or interacting polypeptide or substrate polypeptide.

Thus the substantially pure GSK3 or interacting polypeptide or substrate polypeptide may include a contaminant wherein the contaminant comprises less than 95%, 90%, 80% or 70% of the composition by weight, preferably less than 50% of the composition, (or possibly more preferably less than 30% of the composition, still more preferably less than 10% of the composition and most preferably less than 5%) of the composition by weight.

The substantially pure said GSK3 or interacting polypeptide or substrate polypeptide may be combined with other components *ex vivo*, said other components not being all of the components found in the cell in which said protein kinase or interacting polypeptide or substrate polypeptide is naturally found.

The said GSK3 and optionally said interacting polypeptide may be exposed to each other and to the compound to be tested in a cell in which the said protein kinase and optionally the said interacting polypeptide are both expressed. The GSK3 may be the endogenous protein kinase or it may be a GSK3 expressed from a recombinant construct. Similarly, the said interacting polypeptide may be endogenous or it may be expressed from a recombinant construct. The GSK3 and/or the interacting polypeptide may be GFP fusion proteins, as discussed in Example 2.

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A complex may also be detected by coimmunoprecipitation or copurification experiments, for example in material from cells in which the GSK3 and the said interacting polypeptide are coexpressed.

5   A further aspect of the invention provides a said interacting polypeptide immobilised on a surface of an article suitable for use as a test surface in a surface plasmon resonance method. The surface may be a SensorChip™ surface, for example a SensorChip CM5™ or SA SensorChip™ surface. It is preferred that the interacting polypeptide has fewer than about 400, 380,

10   350, 300, 250, 200, 150, 100, 80, 50, 40 or 30 amino acids.

It is preferred that the interacting polypeptide interacts with the phosphate binding site that is defined by residues including arginine 96 of full-length GSK3β.

15

In relation to the method of any of the previous aspects of the invention, the interacting polypeptide may be part of the GSK3 polypeptide chain. It is preferred in this case that the interaction is an intramolecular interaction.

20   The ability of the compound to inhibit, promote or mimic the interaction of GSK3 with the interacting compound or polypeptide may preferably be measured using scintillation proximity assay techniques or surface plasmon resonance. Suitable techniques are described in Balendran *et al* (1999) *supra* and related patent applications referred to above. Microcalorimetry

25   may also be used with pure components.

A further aspect of the invention provides a method of selecting or designing a compound that modulates the activity of GSK3, the method comprising the step of using molecular modelling means to select or design

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a compound that is predicted to interact with GSK3, wherein a three-dimensional structure of the phosphate binding site of the GSK3 that is defined by residues including arginine 96 of full-length GSK3β and/or with a three-dimensional structure of an interacting polypeptide, for example

5   comprising the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, is compared with a three-dimensional structure of a compound, and a compound that is predicted to interact with the said phosphate binding site is selected.

10

A further aspect of the invention provides a method of selecting or designing a compound that modulates the activity of GSK3, the method comprising the step of using molecular modelling means to select or design a compound that is predicted to interact with GSK3, wherein a three-

15   dimensional structure of an interacting polypeptide comprising the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, is compared with a three-dimensional structure of a compound, and a compound that is predicted to interact with the active site

20   is selected.

A further aspect of the invention provides a method of selecting or designing a compound that modulates the activity of GSK3, the method comprising the step of using molecular modelling means to select or design

25   a compound that is predicted to interact with GSK3, wherein a three-dimensional structure of the GSK3 site, alteration of which modulates activity of GSK3 towards non-phosphorylated substrates, that is defined by residues including leucine 128 of full-length human GSK3β, is compared

with a three-dimensional structure of a compound, and a compound that is predicted to interact with the said substrate binding site is selected.

Thus, the three-dimensional structure of a compound may be compared with the three-dimensional structure of an interacting polypeptide comprising the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof. In particular, the structure of the compound may be compared with the structure of the portion (the interacting portion) of the interacting polypeptide that interacts with the phosphate binding site, as discussed above and in Example 1, for example the portion (for example three amino acids on either or both sides; for example R-T-S/T-Sp-F-A-E) flanking the phosphoserine of the interacting polypeptide. A compound that mimics the structure of the interacting polypeptide, preferably the interacting portion of the polypeptide, still more preferably the features of the interacting portion that interact with residues of GSK3 that define the phosphate binding site, ie residues equivalent to Arg 96 and possibly also Lys 94 of full-length human GSK3β, may be selected.

The three-dimensional structure of a compound may be compared with the three-dimensional structure of the phosphate binding site (and optionally with the three-dimensional structure of a mutated phosphate binding site (which may or may not remain capable of acting as a phosphate binding site), for example a phosphate binding site in which the residue equivalent to Arg 96 of full-length human GSK3β is mutated, for example to Ala or Lys). A compound that can interact with the phosphate binding site, in particular residues equivalent to Arg 96 of full-length human GSK3β, in a similar manner (for example similar separation and/or type of interaction ie hydrophobic or ionic, and/or similar cumulative energy of interaction) to an

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interacting polypeptide may be selected.   Methods of assessing the
interaction are well known to those skilled in the art.

The three-dimensional structures that are compared may be predicted three-
5   dimensional structures or may be three-dimensional structures that have
been determined, for example by techniques such as X-ray crystallography,
as well known to those skilled in the art. The three-dimensional structures
may be displayed by a computer in a two-dimensional form, for example on
a computer screen. The comparison may be performed using such two-
10   dimensional displays.

The three-dimensional structure of GSK3 may be predicted on the basis of
determined structures of PKA and CDKs, as discussed in Example 1. PDB
references for PKA structures include 1ATP, 1CDK; for CDK2 structures,
15   1JST, 1 QMZ.

The following relate to molecular modelling techniques: Blundell *et al*
(1996) Stucture-based drug design *Nature* **384**, 23-26; Bohm (1996)
Computational   tools   for   structure-based   ligand   design
20   *Prog Biophys Mol Biol* **66(3)**, 197-210; Cohen *et al* (1990) *J Med Chem* **33**,
883-894; Navia *et al* (1992) *Curr Opin Struct Biol* **2**, 202-210 .

The following computer programs, for example, may be useful in carrying
out the method of this aspect of the invention: GRID (Goodford (1985) *J*
25   *Med Chem* **28**, 849-857; available from Oxford University, Oxford, UK);
MCSS (Miranker *et al* (1991) *Proteins: Structure, Function and Genetics*
11, 29-34; available from Molecular Simulations, Burlington, MA);
AUTODOCK (Goodsell *et al* (1990) *Proteins: Structure, Function and*
Genetics **8**, 195-202; available from Scripps Research Institute, La Jolla,

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CA); DOCK (Kuntz *et al* (1982) *J Mol Biol* **161**, 269-288; available from the University of California, San Francisco, CA); LUDI (Bohm (1992) *J Comp Aid Molec Design* **6**, 61-78; available from Biosym Technologies, San Diego, CA); LEGEND (Nishibata *et al* (1991) *Tetrahedron* **47**, 8985; available from Molecular Simulations, Burlington, MA); LeapFrog (available from Tripos Associates, St Louis, MO); Gaussian 92, for example revision C (MJ Frisch, Gaussian, Inc., Pittsburgh, PA ©1992); AMBER, version 4.0 (PA Kollman, University of California at San Francisco, ©1994); QUANTA/CHARMM (Molecular Simulations, Inc., Burlington, MA ©1994); and Insight II/Discover (Biosym Technologies Inc., San Diego, CA ©1994). Programs may be run on, for example, a Silicon Graphics™ workstation, Indigo^2™ or IBM RISC/6000™ workstation model 550.

The selected or designed compound may be synthesised (if not already synthesised) and tested for its effect on GSK3, for example its effect on the protein kinase activity. The compound may be tested in a screening method of the invention.

A further aspect of the invention is a compound identified or identifiable by the above selection/design method of the invention.

The ability of the compound to inhibit or promote the interaction of the said GSK3 with the interacting polypeptide or compound may be measured by detecting/measuring the interaction using any suitable method and comparing the interaction detected/measured in the presence of different concentrations of the test compound, for example in the absence and in the presence of the test compound, for example at a concentration of about 100μM, 30μM, 10μM, 3μM, 1μM, 0.1μM, 0.01μM and/or 0.001μM.

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Suitable methods include methods analogous to those discussed above and described in Example 1 and Example 2, for example yeast two-hybrid interactions, co-purification, ELISA, co-immunoprecipitation, scintillation proximity assay, surface plasmon resonance and FRET methods.

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A further aspect of the invention provides a compound capable of modulating the protein kinase activity of GSK3, wherein the compound inhibits the interaction of GSK3 with an interacting polypeptide, wherein the interacting polypeptide interacts with the phosphate binding site of

10 GSK3 that is defined by residues including arginine 96 of full-length GSK3β and/or comprises the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, wherein the compound is not a substrate of GSK3 or a pGS-derivable pseudosubstrate of GSK3, as

15 discussed above (ie as described in Fiol *et al* (1990) *J Biol Chem* 265(11), 6061-6065).

A further aspect of the invention provides a compound capable of modulating the protein kinase activity of GSK3, wherein the compound is

20 capable of interacting with the phosphate binding site of GSK3 that is defined by residues including arginine 96 of full-length GSK3β wherein the compound is not a substrate of GSK3 or (as elsewhere, for the avoidance of doubt) a pGS-derivable pseudosubstrate of GSK3.

25 A still further aspect of the invention provides a compound capable of modulating the protein kinase activity of GSK3, wherein the compound modulates the rate or degree of phosphorylation of a substrate polypeptide of GSK3 by GSK3 in the presence of an interacting polypeptide, wherein the interacting polypeptide comprises the amino acid sequence

RPRTTSpFAESC (NT-Ptide) or TTSpFAESC (NT-Ptide-8) or variant thereof, wherein the compound is not a substrate of GSK3 or a pGS-derivable pseudosubstrate of GSK3.

5   A further aspect of the invention provides a compound capable of inhibiting the protein kinase activity of GSK3 wherein the compound inhibits to a greater degree the rate or degree of phosphorylation by GSK3 of (1) a primed substrate polypeptide of GSK3 than (2) a non-phosphate-dependent , for example non-primed or phosphate-independent substrate of GSK3, as

10   defined above.   The compound is not a substrate of GSK3 or a pGS-derivable pseudosubstrate of GSK3, which may not have such properties.

The GSK3 is preferably active GSK3.

15   A still further aspect of the invention provides a compound identifiable by a screening method of the invention, provided that the compound is not a substrate of GSK3 or a pGS-derivable pseudosubstrate of GSK3.

It is preferred that the compound according to the preceding aspects of the

20   invention is not a pseudosubstrate of GSK3 derivable from a known substrate of GSK3 in which the residue phosphorylatable by GSK3 is replaced by a non-phosphorylatable residue. It will be appreciated that a polypeptide derivable from the N-terminus of GSK3, for example a polypeptide comprising the sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-

25   F-A-E, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, is not such a pseudosubstrate.

It is preferred that the compound is not a polypeptide. It is also preferred that the compound is not a peptidomimetic compound.

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However, the compound may be or comprise a polypeptide having the sequence Sp-F-A-E-$(X)_m$ or T-S/T-Sp-F-A-E-$(X)_m$, for example SpFAESC or SpFAESCor SpFAE$(X)_m$ or TTSpFAESC or TTSpFAE$(X)_m$, for example TTSpFAESC (NT-Ptide-8). Residues $(X)_m$ preferably are derived from the residues of GSK3 C-terminal to the residues corresponding to the S/T-Sp-F-A-E motif, and m may be between 0 and 500, preferably 0 or 1, 2, 3, 4 or 5 to 10, 20, 30, 40, 50, 80, 100 or 200. The polypeptide may consist of or comprise contiguous residues derivable from GSK3. For example, it may comprise the N-terminal about 223, 220, 200, 180, 160, 140, 120, 100, 80, 70, 65, 63, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10 or 5 amino acids of GSK3, or a variant or fusion thereof that preferably has the SpFAESC, SpFAE or T-S/T-Sp-F-A-E sequence. Alternatively, the polypeptide may comprise the motif Sp-F-A-E fused to a linker region and a peptide derived from FRAT or Axin, for example FRATtide (see Thomas *et al* (1999)), to give affinity towards GSK3. Such a polypeptide may inhibit the phosphorylation of pGS without affecting the phosphorylation of GS dephospho-peptides. This polypeptide may prevent interactions between axin and GSK3 and may thereby inhibit its phosphorylation. Such a polypeptide may serve as a proof of concept that it would be possible to inhibit differentially the phosphorylation of a primed polypeptide (GS-P) without affecting the phosphorylation of a non-primed polypeptide (GS). Such a polypeptide may have affinity towards GSK3 through the use of binding sites for Axin and FRAT. Thus, such polypeptides may have overlapping binding sites with Axin and FRAT, and may affect their phosphorylation. It is not known how Tau interacts with GSK3, so it remains a possibility that this kind of polypeptide would inhibit primed-Tau phosphorylation but not the non-primed-Tau phosphorylation.

It will be appreciated that the polypeptide may comprise a covalent modification, for example it may be modified by biotinylation ie comprise a biotin group.

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The compound may be, for example, a compound selected on the basis of, or designed to have, as well known to those skilled in the art, a three-dimensional conformation that may be similar to that of an interacting polypeptide, as discussed above.

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A further aspect of the invention provides a mutated GSK3, wherein one or more residues defining the phosphate binding site of GSK3 that is defined by residues including arginine 96 of full-length GSK3β is mutated. It is preferred that a mutated residue(s) is the residue equivalent to residue

15   arginine 96 or lysine 94 of full-length GSK3β. It is preferred that the residue equivalent to lysine 94 of full-length human GSK3β is mutated to an acidic residue, for example glutamate residue.

A still further aspect of the invention provides a mutated GSK3, wherein

20   one or more residues defining the GSK3 site, alteration of which modulates activity of GSK3 towards non-phosphorylated substrates, (which may be a hydrophobic pocket) that is defined by residues including leucine 128 of full-length human GSK3β is mutated. It is preferred that a mutated residue(s) is the residue equivalent to residue leucine 128 of full-length

25   human GSK3β.

It is preferred that the residue at the position equivalent to residue Arg 96, Lys 94 or Leu 128 of full length human GSK3β is mutated to an Ala (or, in the case of Arg96, Lys).

A further aspect of the invention provides a preparation comprising GSK3, and a second, interacting compound, wherein the interacting compound is capable of interacting with the phosphate binding site of GSK3 that is

5   defined by residues including arginine 96 of full-length GSK3β and/or comprises the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, wherein the interacting compound is not a substrate of GSK3. The preparation may further

10   comprise a substrate polypeptide of GSK3. The preparation may be present in a cell, for example a cell in which a said compound is expressed as a recombinant polypeptide.

We include any composition that includes at least 30% of the protein

15   content by weight as the said GSK3 or interacting polypeptide or (if appropriate) substrate polypeptide (ie in combination), preferably at least 50%, more preferably at least 70%, still more preferably at least 90% and most preferably at least 95% of the protein content is the said protein kinase or interacting polypeptide or (if appropriate) substrate polypeptide.

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Thus, the invention also includes preparations comprising the said GSK3, the said interacting compound, for example polypeptide, and the said substrate polypeptide (if appropriate), and a contaminant wherein the contaminant comprises less than 70% of the composition by weight,

25   preferably less than 50% of the composition, more preferably less than 30% of the composition, still more preferably less than 10% of the composition and most preferably less than 5% of the composition by weight. The invention also includes a preparation comprising the said GSK3 and the said interacting compound, for example polypeptide, and the said substrate

polypeptide (if appropriate) when combined with other components *ex vivo*, said other components not being all of the components found in any cell in which said GSK3 and/or interacting compound, for example polypeptide, and/or substrate polypeptide may naturally be found.

A further aspect of the invention provides a method of modulating the protein kinase activity of GSK3 wherein the GSK3 is exposed to a compound or composition of the invention. It is preferred that the compound or composition inhibits to a greater degree the rate or degree of phosphorylation by GSK3 of (1) a primed substrate polypeptide of GSK3 than (2) a non-phosphate-dependent, for example phosphate-independent or non-primed substrate of GSK3.

A further aspect of the invention provides a method of modulating in a cell the protein kinase activity of GSK3, wherein a recombinant interacting polypeptide is expressed in the cell, wherein the interacting polypeptide interacts with the phosphate binding site of GSK3 but does not prevent phosphorylation of non-phosphate-dependent substrates of GSK3. Examples of such a peptide are discussed above. The interacting polypeptide may be expressed as a fusion protein.

Suitably, the method comprises the steps of providing a recombinant polynucleotide suitable for expressing the interacting polypeptide in the cell, providing the recombinant polynucleotide in the cell, and exposing the cell to conditions under which the cell expresses the interacting polypeptide from the recombinant polynucleotide.

A further aspect of the invention provides a recombinant cell comprising GSK3 and a second, recombinant interacting polypeptide as defined above.

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The GSK3 may also be recombinant. The GSK3 and/or interacting polypeptide may be fusion proteins, for example as discussed in Example 2. Such a cell may be useful in a screen for detecting compounds that are capable of modulating the interaction between the GSK3 and the interacting

5   polypeptide, for example by detecting modulation of FRET between chromophores (for example GFPs) attached to the GSK3 and interacting polypeptide.

A further aspect of the invention provides an interacting polypeptide as

10   defined above. Preferably the interacting polypeptide interacts with the phosphate binding site of GSK3 but does not prevent phosphorylation of non-phosphate-dependent substrates of GSK3. Examples of such a peptide are discussed above. In particular, such an interacting polypeptide may have the amino acid sequence T/S-T-Sp-F-A-E(X)$_m$ (where m is as defined

15   above), for example TTSpFAESC (NT-Ptide-8). The interacting polypeptide is not a pGS-derived pseudosubstrate of GSK3 (for example as described in Fiol *et al* (1990) *supra*), as discussed above.

The said polypeptide of the invention may comprise, for example, a GST

20   portion, as described in Example 1 or GFP portion. This may be useful in purifying and/or detecting the said polypeptide.

A further aspect of the invention provides a polynucleotide encoding a polypeptide or mutated GSK3 of the invention. A still further aspect of the

25   invention provides a recombinant polynucleotide suitable for expressing a polypeptide or mutated GSK3 of the invention. A yet further aspect of the invention provides a host cell comprising a polynucleotide of the invention.

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A further aspect of the invention provides a method of making a polypeptide or mutated GSK3 of the invention, the method comprising culturing a host cell of the invention which expresses said polypeptide or mutated GSK3 and isolating said polypeptide or mutated GSK3.

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A cell which expresses a mutated GSK3 of the invention, for example GSK3R96A or GSK3R96K, may also have the storage of metabolic energy modified and may therefore also be useful because of this.

10   Such a cell may also be useful in investigating/validating drugs directed to the GSK3 phosphate binding site.   A GSK3-/- cell line transfected with GSK3 wt or mutant may be useful in providing evidence that that compound is acting against the GSK3 phosphate binding site. The phenotype of the GSK3 wt transfected cells in the presence of the compound may be

15   predicted to be similar to that of GSK3 96A or GSK 96K (for example) transfected cells (in the absence of the compound).   In addition, the compound should not affect the characteristics of the GSK3 96A or GSK3 96K (or other phosphate binding site-disrupted mutant) transfected cells.

20   A further aspect of the invention provides a polypeptide or mutated GSK3 obtainable by the above method.

The interacting polypeptide as defined above may have up to about 950, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80, 70, 60, 50, 40, 30, 20, 18,

25   16, 15, 14, 12, 10, 8 or 7 amino acids residues.   It will be appreciated that the polypeptide may comprise a covalent modification, for example it may be modified by biotinylation ie comprise a biotin group. Such a peptide may be useful in the methods of the invention, for example in altering the enzymic activity of GSK3 *in vitro* or *in vivo*.

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The above polypeptides may be made by methods well known in the art and as described below and in Example 1, for example using molecular biology methods or automated chemical peptide synthesis methods.

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It will be appreciated that peptidomimetic compounds may also be useful. Thus, by "polypeptide" or "peptide" we include not only molecules in which amino acid residues are joined by peptide (-CO-NH-) linkages but also molecules in which the peptide bond is reversed. Such retro-inverso

10   peptidomimetics may be made using methods known in the art, for example such as those described in M9zilIre et al (1997) J. Immunol. 159, 3230-3237, incorporated herein by reference. This approach involves making pseudopeptides containing changes involving the backbone, and not the orientation of side chains. M9zilIre et al (1997) show that, at least for

15   MHC class II and T helper cell responses, these pseudopeptides are useful. Retro-inverse peptides, which contain NH-CO bonds instead of CO-NH peptide bonds, are much more resistant to proteolysis.

Similarly, the peptide bond may be dispensed with altogether provided that

20   an appropriate linker moiety which retains the spacing between the CI atoms of the amino acid residues is used; it is particularly preferred if the linker moiety has substantially the same charge distribution and substantially the same planarity as a peptide bond.

25   It will be appreciated that the peptide may conveniently be blocked at its N- or C-terminus so as to help reduce susceptibility to exoproteolytic digestion.

Thus, it will be appreciated that the interacting polypeptide may be exposed may be a peptidomimetic compound, as described above.

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A further aspect of the invention provides a cell containing a recombinant nucleic acid suitable for expressing GSK3 (for example an Arg96 or Leu128 mutant) and a recombinant nucleic acid suitable for expressing an
5   interacting polypeptide, as defined above.

The cell is preferably a mammalian or insect cell, but may alternatively be a bacterial (for example *E. coli*) or yeast cell.

10   A further aspect of the invention provides an antibody that interacts with the phosphate binding site of GSK3 that is defined by residues including arginine 96 of full-length GSK3β or with the site of GSK3, alteration of which modulates activity of GSK3 towards non-phosphorylated substrates, that is defined by residues including leucine 128 of full-length human
15   GSK3β. The antibody may interact with an epitope comprising the amino acid sequence of GSK3 flanking the residue equivalent to arginine 96 (and optionally lysine 94) or leucine 128 of full-length GSK3β.

Antibodies reactive towards the said polypeptides may be made by methods
20   well known in the art. In particular, the antibodies may be polyclonal or monoclonal.

Suitable monoclonal antibodies which are reactive towards the said polypeptide may be prepared by known techniques, for example those
25   disclosed in *"Monoclonal Antibodies: A manual of techniques"*, H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and Applications", SGR Hurrell (CRC Press, 1982).

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Techniques for preparing antibodies are well known to those skilled in the art, for example as described in Harlow, ED & Lane, D "Antibodies: a laboratory manual" (1988) New York Cold Spring Harbor Laboratory.

5   It will be appreciated that the invention provides screening assays for drugs which may be useful in modulating, for example either enhancing or inhibiting, the protein kinase activity of GSK3. The compounds identified in the methods may themselves be useful as a drug or they may represent lead compounds for the design and synthesis of more efficacious

10   compounds.

The compound may be a drug-like compound or lead compound for the development of a drug-like compound for each of the above methods of identifying a compound. It will be appreciated that the said methods may be

15   useful as screening assays in the development of pharmaceutical compounds or drugs, as well known to those skilled in the art.

The term "drug-like compound" is well known to those skilled in the art, and may include the meaning of a compound that has characteristics that

20   may make it suitable for use in medicine, for example as the active ingredient in a medicament. Thus, for example, a drug-like compound may be a molecule that may be synthesised by the techniques of organic chemistry, less preferably by techniques of molecular biology or biochemistry, and is preferably a small molecule, which may be of less than

25   5000 daltons. A drug-like compound may additionally exhibit features of selective interaction with a particular protein or proteins and be bioavailable and/or able to penetrate cellular membranes, but it will be appreciated that these features are not essential.

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The term "lead compound" is similarly well known to those skilled in the art, and may include the meaning that the compound, whilst not itself suitable for use as a drug (for example because it is only weakly potent against its intended target, non-selective in its action, unstable, difficult to
5   synthesise or has poor bioavailability) may provide a starting-point for the design of other compounds that may have more desirable characteristics.

It will be appreciated that screening assays which are capable of high throughput operation may be particularly preferred. Examples may include
10   cell based assays and protein-protein binding assays. An SPA-based (Scintillation Proximity Assay; Amersham International) system may be used. For example, beads comprising scintillant and a substrate polypeptide or interacting polypeptide may be prepared. The beads may be mixed with a sample comprising ^{32}P- or ^{33}P-γ-labelled GSK3 (as defined above) and
15   with the test compound. Conveniently this is done in a 96-well format. The plate is then counted using a suitable scintillation counter, using known parameters for ^{32}P or ^{33}P SPA assays. Only ^{32}P or ^{33}P that is in proximity to the scintillant, i.e. only that bound to the substrate or interacting polypeptide that is bound to the beads, is detected. Variants of such an
20   assay, for example in which the substrate or interacting polypeptide is immobilised on the scintillant beads *via* binding to an antibody or antibody fragment, may also be used.

A further aspect of the invention is a kit of parts useful in carrying out a
25   method, for example a screening method, of the invention. Such a kit may comprise at least two of (1) GSK3 which is not a mutated GSK3 according to the invention, (2) a mutated GSK3 according to the invention and/or (3) a separate interacting polypeptide wherein the interacting polypeptide interacts with the phosphate binding site of GKS3 and/or comprises the

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amino acid sequence NTP-tide (NTP-tide-11), and is not a substrate of GSK3. The kit may further comprise a primed substrate and/or a non-phosphate-dependent substrate.

5   Further preferences for the GSK3, substrate polypeptide and interacting polypeptide are as indicated above.

It will be understood that it will be desirable to identify compounds that may modulate the activity of GSK3 *in vivo*. Thus it will be understood that

10   reagents and conditions used in the method may be chosen such that the interactions between, for example, the said GSK3 and the interacting polypeptide, are substantially the same as between human GSK3 and the phosphorylated N-terminus of GSK3. It will be appreciated that the compound may bind to the GSK3, for example the phosphate binding site of

15   GSK3, or may bind to an interacting polypeptide, for example the phosphorylated N-terminus of GSK3.

The compounds that are tested in the screening methods of the assay or in other assays in which the ability of a compound to modulate the protein

20   kinase activity of GSK3 may be measured, may be compounds that have been selected and/or designed (including modified) using molecular modelling techniques, for example using computer techniques, as indicated above.

25   A still further aspect of the invention provides a compound (or polypeptide or polynucleotide or mutated GSK3) of the invention for use in medicine.

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A sill further aspect of the invention provides a pharmaceutical composition comprising a compound (or polypeptide or polynucleotide or mutated GSK3) of the invention and a pharmaceutically acceptable excipient.

5   The compound (or polypeptide or polynucleotide) may be administered in any suitable way, usually parenterally, for example intravenously, intraperitoneally or intravesically, in standard sterile, non-pyrogenic formulations of diluents and carriers. The compound (or polypeptide or polynucleotide) may also be administered topically, which may be of

10   particular benefit for treatment of surface wounds, hair loss or baldness. The compound (or polypeptide or polynucleotide) may also be administered in a localised manner, for example by injection. The compound may be useful as an antifungal (or other parasitic, pathogenic or potentially parasitic or pathogenic organism) agent.

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A further aspect of the invention is the use of a compound (or polypeptide or polynucleotide) as defined above in the manufacture of a medicament for the treatment of a patient in need of modulation of signalling by GSK3, for example insulin signalling. The patient may be in need of inhibition of

20   phosphorylation of primed substrates of GSK3 but not in need of inhibition of phosphorylation of non-phosphate-dependent substrates of GSK3, for example axin, β-catenin or Tau. It will be appreciated that inhibition of phosphorylation of non-phosphate-dependent substrates of GSK3 may generally be undesirable, in that such inhibition (particularly of axin or β-

25   catenin) may promote oncogenesis.

A further aspect of the invention is a method of treating a patient in need of modulation of signalling by GSK3, wherein the patient is administered an

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effective amount of a compound (or polypeptide or polynucleotide) as defined above.

A compound that is capable of inhibiting the phosphorylation of primed
5   substrates of GSK3 may be useful in the treatment of Alzheimer's disease and/or diabetes.

As noted above, insulin leads to the activation of glycogen synthase, which is the key enzyme in the conversion of glucose into glycogen. In diabetic
10   patients, there is a failure to produce or respond correctly to insulin. Since insulin inhibits GSK3 (through the PKB-mediated phosphorylation of Ser9 of GSK3β for example), a compound which mimics the action of insulin, for example an inhibitor of GSK3, might be useful in the treatment of diabetes. While this could be achieved using a general GSK3 inhibitor, there may be
15   great benefits from using a selective compound such as the kind identified by our screening methodologies (a phosphate binding site-binding compound), since it would not be predicted to inhibit β-catenin phosphorylation, thereby leading to accumulation of β-catenin and possibly to cancer, most likely of epithelial tissues such as colon and skin.
20

A compound that is capable of decreasing the activity of GSK3, particularly towards primed substrates may be useful in treating diabetes or obesity.

It has recently been suggested that Wnt signalling (inhibition of GSK3) may
25   control the cell fate decision leading to muscle or fat. (Adipocytes and myocytes originate from the same precursor cell). In preadipocytes, lithium (an inhibitor of GSK3) prevents differentiation into mature fat cells. It appears that active Wnt signalling is required for continued commitment to the myocyte lineage, and inhibition of Wnt signalling (activation of GSK3)

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in preadipocytes and myoblasts induces adipogenesis. This appears to occur through the axin / β-catenin substrates of GSK3. A general GSK3 inhibitor or preferential inhibitor of non-phosphate-dependent substrates such as axin and β-catenin may mimic lithium, and keep preadipocytes in their undifferentiated state, which may be useful for stem cell technology by keeping these cells in a pluripotent state. This may also force the non-committed common precursor cell down the muscle cell lineage, which may be desirable, at least in some circumstances.

A compound directed towards the phosphate binding site may not affect this pathway. Thus, such a compound may have the advantage over traditional GSK3 inhibitors that, for example when being used for treatment of a separate condition, it would not be expected to interfere with the differentiation program of these cell lineages.

Increase in the activity of GSK3 may promote apoptosis and may therefore be useful in treating cancer. Conditions in which aiding apoptosis may be of benefit may also include resolution of inflammation.

Published data suggests that inhibition of GSK3 would lead to accumulation of β-catenin which has been proven to be oncogenic in epithelial tissues such as colon and skin. Naturally-occurring mutations of β-catenin which remove the GSK3 sites account for a significant percentage of colon tumours (Polakis P et al (1999) Adv Exp Med Biol 470, 23-32). Mutations in APC (adenomatous polyposis coli) are found in colon cancer patients and this confers a natural predisposition to developing this tumour type. This protein gets its name from the familial disease, FAP (familial adenomatous polyposis coli), and is found in the cell in a complex with GSK3, axin and beta-catenin (and others). It is also a substrate of GSK3, and one of its roles

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in the cell is to facilitate the degradation of β-catenin. It is not clear whether APC is a primed or non-primed substrate of GSK3, or both. Mutations in APC are found in the majority of colon tumours, both familial and sporadic cases. Hence, inhibition of GSK3 may lead to an accumulation of β-catenin

5   and promotion of cancer.

However, there may be circumstances in which it may be favourable to promote apoptosis in cancer cells by inhibiting GSK3 (perhaps in a specific organ), and there is evidence, for example in the liver that loss of GSK3

10   actually leads to apoptosis. This contrasts with the data discussed below in neurones where inhibition of GSK3 prevents apoptosis. The exact pathways and substrates of GSK3 responsible for this effect have not been elucidated, although in the case of the liver apoptosis, NF-kappaB may be involved as a downstream component of GSK3 (Hoeflich *et al* (2000) *Nature* 406(6791),

15   86-90). Thus, a general GSK3 inhibitor or a selective (e.g.phosphate binding site) compound may be useful in the treatment of at least some cancers and of inflammation.

One of the main pathogenic features of Alzheimer's brains are

20   neurofibrillary tangles. These are composed of hyperphosphorylated tau, which is a substrate of GSK3, as well as several other kinases. The inappropriate activation or lack of appropriate inhibition/regulation of GSK3 is therefore implicated in the pathogenesis of this disease. This may occur through naturally-occurring mutations of other components of this complex

25   in neuronal cells which are involved in regulating the interaction/ phosphorylation of tau by GSK3, for example many Alzheimer's patients carry mutations in the presenilin gene, which has been demonstrated to be causative of the disease. A phosphate binding site-binding compound may be more useful than a general GSK3 inhibitor in this treatment. The basis for

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this is that although non-primed tau is a substrate of GSK3, a primed form of tau is a 50x better substrate. In tau there appear to be some GSK3 sites which require priming and others which do not. The contribution of these two types of sites to the hyperphosphorylated tau that makes up the neurofibrillary tangles in Alzheimer's brains is not yet clear.

There are several reports of GSK3 being important for neuronal survival. In other words, inhibition of GSK3 appears to be able to protect neurones from apoptosis.

A compound that is capable of inhibiting GSK3, for example the phosphorylation of primed substrates of GSK3 may suppress or inhibit apoptosis, at least in certain tissues, which may aid cell survival during or following cell damaging processes. It is believed that such compounds are useful in treating disease in which apoptosis is involved. Examples of such diseases include, but are not limited to, mechanical (including heat) tissue injury or ischaemic disease, for example stroke, neural injury and myocardial infarction.

Thus the patient in need of modulation of the activity of GSK3 may preferably be a patient with diabetes.

Thus, a further aspect of the invention provides a method of treating a patient with diabetes the method comprising administering to the patient an effective amount of a compound identified or identifiable by the screening methods of the invention.

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The patient may be a patient with cancer, or a patient in need of inhibition of apoptosis, for example a patient suffering from tissue injury or ischaemic injury, including stroke.

5   Thus, a further aspect of the invention provides a method of treating a patient with an ischaemic disease the method comprising administering to the patient an effective amount of a compound identified or identifiable by the screening methods of the invention.

10   A still further invention provides a use of a compound identifiable by the screening methods of the invention in the manufacture of a medicament for treating an ischaemic disease in a patient.

      If the patient is a patient in need of promotion of apoptosis, for example a
15   patient with cancer, it may be preferred that the compound of the invention that is used in the preparation of the medicament is capable of increasing the activity of GSK3.

      If the patient is a patient with diabetes, it is preferred that the compound of
20   the invention that is used in the preparation of the medicament is capable of decreasing the activity of GSK3, particularly towards primed substrates, for example glycogen synthase. It is particularly preferred that the compound is capable of decreasing the activity of GSK3 towards primed substrates but is not capable of decreasing the activity of GSK3 towards non-phosphate-
25   dependent substrates, for example axin, β-catenin or Tau (or some sites thereof).

      For a patient in need of inhibition of apoptosis, for example a patient with ischaemic disease it may be preferred that the compound of the invention

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that is used in the preparation of the medicament is capable of decreasing the activity of GSK3, particularly towards primed substrates.

5   A further aspect of the invention provides a method of determining whether a patient is predisposed towards a defect in GSK3 signalling, for example a defect in control of glycogen metabolism, for example diabetes, comprising the step of determining whether the patient has a mutation of a residue defining the phosphate binding site of a GSK3 that is defined by residues including arginine 96 of full-length GSK3β or of a residue defining the

10   GSK3 site, alteration of which modulates activity of GSK3 towards non-phosphorylated substrates, that is defined by residues including leucine 128 of full-length human GSK3β. It is preferred that it is determined whether the residue equivalent to arginine 96 of full-length GSK3β is mutated, as discussed further in Example 3.

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The invention will now be described by reference to the following Examples and Figures:

Figure Legends

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Figure 1

Prediction of GSK3 residues involved in the interaction with +4 phosphate present in some GSK3 substrates. The ribbon structures of the kinase domain of PKA (A) and CDK2 (B) bound to ATP are shown. Both

25   structures are in active conformations, phosphorylated in the activation loop (not shown). The specific protein kinase A inhibitor (PKI) backbone and the CDK2 peptide' substrates (shown as sticks) are superimposed on the structures. Based on the assumption that the substrate will bind in a similar manner in GSK3, the region within the box would contain the putative

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phosphate binding site. Major constituents of this region are the αl helix (equivalent to αC in PKA) and the activation loop; residues N90 from PKA and R50 from CDK2, equivalent to R96 in GSK3β, are also represented by sticks. The position of the PSTAIRE motif, involved in CDK2 interaction

5   with cyclin is marked. The inactive (non-phosphorylated) CDK2 structure shows important changes in the position of the activation loop, as well as changes in the αl helix from those shown here. C. Alignment of the amino acid residues of human GSK3β around the αl helix, and the equivalent regions in mouse PKA and human CDK2. Identical residues are denoted by

10   white letters on a black background, and similar residues by grey boxes. Arg96 in GSK3β mutated in this study is marked with an asterisk.

Figure 2

Effect of mutation of residues predicted to form a phosphate binding

15   **site in GSK3β on the specificity for primed versus non-primed substrates.** Wild-type or mutant forms of hGSK3β were expressed in 293 cells and purified by affinity chromatography on glutathione-Sepharose beads. Each protein was incubated for 15 minutes at 30 °C with substrate plus Mg-ATP in the presence or absence of 50mM LiCl or 100nM Ro 31-

20   8220. The assays shown were performed in duplicate and similar results were obtained in three additional independent purifications. A. Phosphorylation of pGS peptide. Specific activities for the wild-type enzyme are shown above the first bar of the graph. All other activities are expressed as a percentage of wild-type activity. B. Phosphorylation of GS

25   peptide. Specific activities for the wild-type enzyme are shown above the first bar of the graph. All other activities are expressed as a percentage of wild-type activity. Inset: representative autoradiograph showing results for wild-type GSK3, quantified below in the first three columns of the graph.

Assays were run on SDS polyacrylamide gels and quantified by phosphorimager. GSK3 activity is measured as incorporation of [γ-^{32}P]ATP into GS peptide only, and does not include GSK3 autophosphorylation. C. Axin [275-510] phosphorylation. D.

5   Quantification of autoradiograph in C. Activities are expressed as a percentage of the activity of the wild-type enzyme.

Figure 3

Effect of NT-Ptide on wild-type GSK3 and GSK3β[R96A] mutant. A.

10   Wild-type GSK3β was assayed against the pGS peptide (10μM) in the absence or presence of various concentrations of NT-Ptide, NT-tide or a control phosphopeptide. Activity was expressed as a percentage of that obtained in the absence of any competing peptide, set at 100%. Errors between duplicates were less than 1%. B. Wild-type and mutant GSK3β

15   enzymes were assayed against the pGS peptide in the absence or presence of 0.4mM NT-Ptide, NT-tide or a control phosphopeptide. Activity is expressed as a percentage of the activity for that enzyme in the absence of any competing peptide. C. Wild-type and mutant GSK3β enzymes were assayed for activity towards Axin [2 75-510], in the absence or presence of

20   0.4mM NT-Ptide, NT-tide or a control phosphopeptide. All assays shown were performed in duplicate and similar results were obtained in two additional independent purifications.

Figure 4

25   **Effect of PKB on GSK3β can be titrated by addition of increasing amounts of primed substrate.** Wild-type GSK3β was pre-incubated with PKB or dilution buffer for 10 minutes at 30°C and then incubated for a further 10 minutes with various concentrations of pGS peptide substrate as indicated. Activity was expressed as a percentage of the activity obtained at

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a given peptide concentration in the absence of PKB (set at 100%). Thus at 20μM pGS peptide, maximal inhibition by PKB was achieved, which was lost with successive increases in the primed substrate. The errors between samples were less than 1% for assays performed in quadruplicate.

Figure 5

GSK3β[R96A] is resistant to inhibition by PKB. Wild-type or mutant GSK3β enzymes were pre-incubated with PKB for 10 minutes at 30°C, then assayed for activity towards the pGS peptide (20μM). Activities are expressed as a percentage of the activity of that enzyme in the absence of PKB (all set at 100%). The assays shown were performed in quadruplicate and similar results were obtained in two additional experiments with proteins from independent purifications.

Figure 6

Model of GSK3 regulation. Wnt signalling leads to the dissociation of Axin from the GSK3 complex due to the binding of FRAT, and thereby inhibits phosphorylation of Axin and β-catenin. This in turn leads to accumulation of β-catenin, and activation of β-catenin-dependent transcription. On the other hand, insulin or growth factors, such as EGF, activate kinases including PKB, which phosphorylate GSK3β at Ser9 at its amino terminus. This inhibits the catalytic activity of the enzyme, leading to activation of glycogen synthase, and other primed substrates. The two distinct mechanisms for inactivation of GSK3 could operate alone or in combination to facilitate entirely different cellular responses in the context of embryonic development, normal physiological processes in the adult or in tumourigenesis.

Figure 7: Possible locations of GFPs in GKS3β

Figure 8. Mutation of Arg96 to Ala or Lys in GSK3β abolishes activity towards primed substrates, but not non-primed substrates. Specific activities

5   for the wild-type enzyme towards the primed pGS and peIF2B peptides were 415 U/mg and 925 U/mg respectively, and for the non-primed GS and eIF2B peptides were 1.33 U/mg and 0.3 U/mg respectively. The sequence of these peptides is given in Methods. The activity of the Arg96Ala (R96A) and Arg96Lys (R96K) mutants were expressed as a percentage of wild-type

10 (WT) activity, which was set at 100%.

Figure 9. The GSK3 Arg96Ala mutant is resistant to inhibition by PKB. **A.** Wild-type (WT) or mutant GSK3 (R96A) enzymes, either unphosphorylated or maximally phosphorylated with PKB, were assayed for activity towards the primed pGS peptide. Activities are expressed as a percentage of the

15 activity of unphosphorylated GSK3 (100%). **B.** The inhibition of GSK3β by PKB is lost progressively as the concentration of primed pGS peptide substrate is increased. Activity is expressed as percentage inhibition of the activity of unphosphorylated GSK3.

Figure 10. Effect of peptides derived from the N-terminus of GSK3β on the

20 activity of wild-type GSK3β. **A.** The activity of wild-type GSK3 towards the pGS peptide was measured in the absence or presence of NTptide-11,

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NTtide-11 or an unrelated control phospho-peptide (see Methods for sequences). Duplicate determinations varied less than 1%. B. NTptide-8 selectively inhibits the activity of wild-type GSK3β towards primed substrates. The activity of wild-type GSK3 towards primed and non-primed

5   substrates was measured in the absence or presence of NTptide-11 or NTptide-8 (both at 2mM). Activity was expressed as a percentage of that obtained in the absence of either peptide.

Figure 11. Mutation of Leu128 to Ala in GSK3β abolishes activity towards Axin. Wild-type GSK3 and the L128A mutant were tested for their ability to

10   phosphorylate the primed pGS peptide substrate (A) or Axin (B). The activity of the Leu128Ala (L128A) mutant was expressed as a percentage of wild-type (WT) activity, which was set at 100%. For the Axin assays, the reactions were loaded onto SDS-polyacrylamide gels, exposed to autoradiographic film (B, lower panel) and quantified by phosphorimager

15   analysis (B, upper panel).

Figure 12. Model for the regulation of GSK3. Following the binding of Wnt to its receptor, Axin is displaced from GSK3 as a result of binding of FRAT to GSK3, which leads to the stabilisation of β-catenin and its accumulation in the nucleus where it stimulates the transcription of Wnt target genes. The

20   residues on GSK3 forming part of the high affinity interaction site for FRAT and Axin are not known. FRAT binding does not affect the phosphorylation

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of primed substrates of the insulin signalling pathway. In contrast, insulin

induces the activation of PKB, which phosphorylates GSK3β at Ser9. The

phosphorylated N-terminus then inhibits GSK3 by acting as a

pseudosubstrate, competing with primed substrates for the phosphate-

5   binding and catalytic sites on GSK3. This leads to the dephosphorylation

and activation of glycogen synthase and eIF2B, and hence to the stimulation

of glycogen and protein synthesis. GSK3 bound to Axin is not available for

phosphorylation on Ser9 in response to insulin restricting the effects of

insulin to a specific subset of GSK3 substrates. A compound that interacts

10   with the phosphate-binding site on GSK3 would selectively inhibit the

phosphorylation of glycogen synthase and eIF2B without affecting the

phosphorylation of Axin and β-catenin. In contrast, compounds that target

the ATP binding site would mimic the effects of Wnt as well as insulin.

Figure 13. BiaCore analysis of NTPtide-11 binding to GST-GSK3β.

15   Binding was analysed directly by surface plasmon resonance (SPR) in a

BiaCore 3000 system. Biotin-NTPtide-11 was bound (100RUs) to a

streptavidin-coated Sensor chip SA (Biacore AB, Stevenage, UK) following

manufacturer's instructions. The system was equilibrated in a buffer

containing 50 mM Hepes pH7.5, 150 mM NaCl, 0.005% polysorbate and

20   wild type GST-GSK3β (100 nM) was injected at a flow rate of 30 μl/min.

The specific binding (arbitrary response units, RUs) is expressed as the

difference in binding with respect to a lane uncoupled to the specific

peptide.

Figure 14. NTPtide-11/Axin based kinase assay to screen for compounds

that can bind to the phosphate binding site of GSK3. The ability of wild-

type GSK3β to phosphorylate Axin was determined in 20µl assays

containing 50 mM Tris-HCl (pH 7.5), 0.1% 2-mercaptoethanol, 10 mM

MgCL$_2$, 100 µM [γ-^{32}P] ATP (500 cpm/pmol), GSK3β and Axin [275-510]

(1.8µM), in the presence or absence of NTPtide-11 (500 µM) and

phosphoserine (10mM) as indicated. In reactions containing phosphoserine,

GSK3 was first pre-incubated with phosphoserine for 15 minutes prior to

the addition of NTPtide-11, Axin and ATP. After incubation for 15 minutes

at 30ºC, reactions were stopped by the addition of SDS, heated at 90ºC for

5 minutes, then loaded onto SDS-polyacrylamide gels. The bands were then

quantified by phosphorimager analysis.

Example 1: A common phosphate binding site explains the unique
specificity of GKS3 and its inactivation by phosphorylation

Glycogen synthase kinase-3 (GSK3) is a serine/threonine kinase with two

distinct functions. First, its activity towards glycogen synthase and

eukaryotic protein synthesis inhibitor-2B (eIF2B) is inhibited by insulin via

the protein kinase B (PKB)-catalysed phosphorylation of Ser21 (GSK3α)

and Ser9 (GSK3β), resulting in the stimulation of glycogen and protein

synthesis. Secondly, it participates in the regulation of Axin and β-catenin levels in the Wnt signalling pathway that specifies cell fate during embryonic development. The activity of GSK3 towards glycogen synthase and eIF2B requires the presence of phosphoserine at n+4, where n is the site of phosphorylation, while the phosphorylation of Axin and β-catenin are not known to be dependent on a "primed" phosphate and appear to rely on high affinity interactions in a multi-protein complex with GSK3. Here we provide evidence for the existence of a phosphate binding site in GSK3β, which is involved in the interaction with the "primed phosphate" of substrates, as well as with phosphorylated Ser9. Thus, mutation of Arg96 to Ala abolished the ability of GSK3β to phosphorylate "primed" substrates, but had no effect on activity towards non-primed substrates, such as Axin. The same mutation also prevented the inhibition of GSK3β resulting from the phosphorylation of Ser9. Consistent with a single phosphate binding site, the extent of inhibition of GSK3β decreased with increasing concentrations of the "primed" substrate, and a phosphopeptide corresponding to residues 4-14 of GSK3β inhibited activity very specifically. We propose that the phosphorylated N-terminal region folds back on the catalytic core as a pseudosubstrate, with the phosphorylated Ser9 occupying the same phosphate binding site used by "primed" substrates. The identification of this key interaction site suggests new opportunities for the design of more selective GSK3 inhibitors with therapeutic potential for the treatment of diabetes and other diseases.

Materials and Methods

Materials

Complete protease inhibitor cocktail tablets were from Roche; tissue culture reagents were from Life Technologies and glutathione-Sepharose was from

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Amersham Pharmacia Biotech. Ro 31-8220 was purchased from Calbiochem.

Peptides

The pGS (also called GS-2) and GS peptides were based on the sequence in glycogen synthase phosphorylated by GSK3 (YRRAAVPPSPSLSRHSSPHQSpEDEEE and YRRAAVPPSPSL-SRHSSPHQAEDEEE, where Sp is phosphoserine). The peIF2B (RRAAEELDSRAGSpPQL) and eIF2B (RRAAEELDSRAGSPQL) peptides were derived from the GSK3 site in eIF2B (Welsh and Proud, (1993) *Biochem. J.* **294**, 625-629). The NTptide-11 (RPRTTSpFAESC), NTtide-11 (RPRTTSFAESC), [P/S]NTptide-11 (RSRTTSpFAESC) and NTptide-8 (TTSpFAESC) peptides were all based on the sequence of the N-terminus of GSK3β. The control phospho-peptide had the sequence QGDLMTpPQFTP, where Tp is phospho-threonine. All peptides were synthesised by Dr G.Bloomberg (University of Bristol, UK).

General methods

Molecular biology techniques were performed using standard procedures. Site directed mutagenesis was performed using a QuikChange Kit (Stratagene) following the instructions provided by the manufacturer. DNA constructs used for transfection were purified from bacteria using a Qiagen plasmid Mega kit according to the manufacturer's protocol, and their sequence verified using an automated DNA sequencer (Model 373, Applied

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Biosystems). Human embryonic kidney (HEK)293 cells were cultured on 10cm dishes in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum.

5   *Purification of human GSK3β proteins*

Human GSK3β (wild-type and mutant) proteins were expressed in 293 cells with an N-terminal GST tag from the pEBG-2T vector [19], and affinity purified on glutathione-Sepharose [20]. Approximately 0.1-0.2mg of each GST fusion protein was obtained by transfection of ten 10cm diameter

10   dishes of HEK293 cells and each protein was about 50% pure as judged by SDS-PAGE (data not shown). Human Axin [275-510] was expressed in bacteria with a GST tag at the N-terminus from the pGEX 4T-2 vector, and purified as described previously [21].

15   Glycogen synthase was purified from rabbit skeletal muscle as described previously (Nimmo *et al* (1976) *Eur. J. Biochem.* **68**, 21-30). PKBα was expressed as a hexahistidine-tagged protein in insect cells, purified on nickel/nitriloacetate-agarose, and then activated to a specific activity of 310 U/mg by incubation with MAPKAPK2 and PDK1.

20

Measurement of GSK3 activity

The ability of wild-type and mutant GSK3β to phosphorylate the synthetic peptides, pGS or GS, and Axin [275-510] was determined in 20µl assays

25   containing 50mM Tris-HCl pH 7.5, 0.1% 2-mercaptoethanol, 10 mM MgCl$_2$, 100µM [γ^{32}P]ATP (-500 c.p.m./nmol), GSK3β and either Axin

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[275-510] (1.8μM), glycogen synthase (0.6μM), the primed pGS peptide (20μM), the primed peIF2B peptide (20μM), the non-primed GS peptide (1.5mM), the non-primed eIF2B peptide (3mM) or the [P/S]NTptide-11 peptide (20μM). After incubation for 15 min at 30°C, the reaction was

5　stopped. For the pGS, peIF2B and [P/S]NTptide-11 peptide assays, this was done by addition of 20μl of 150mM phosphoric acid, then 35μl of the mixture was spotted onto P81 phosphocellulose paper and the papers were washed and analysed as described previously for assays of MAP kinase [22]. For the non-primed GS and eIF2B peptide assays, and assays using

10　Axin [275-510] or glycogen synthase, the reactions were stopped by addition of SDS, heated at 90°C for 5 min and loaded onto SDS-polyacrylamide gels. GSK3 activity was then quantified by phosphorimager analysis. The N-terminal phosphopeptide (NT-Ptide; NT-Ptide-11), the N-terminal dephosphopeptide (NT-tide; NT-tide-11)), the short version of

15　NTptide-11 (NTptide-8) or the control phosphopeptide were included in the reactions as indicated. Control assays were carried out in parallel in which either GSK3β or peptide substrate were omitted; these values were always less than 5% of the activity measured in the presence of these reagents.

20　Inactivation of GSK3β (by maximally phosphorylating GSK3 with PKB) was performed by pre-incubating the GSK3β in a 20μl assay consisting of 50mM Tris-HCl pH 7.5, 0.1% 2-mercaptoethanol, 10mM $MgCl_2$, 100μM cold ATP, GSK3β (0.1μM) and PKBα (86nM) for 10 min at 30°C. Then 20μl pGS peptide reaction mixture (50mM Tris-HCl pH 7.5, 0.1% 2-

25　mercaptoethanol, 10mM $MgCl_2$, 100μM [γ-^{32}P]ATP (-500 c.p.m./pmol) and pGS peptide at the concentrations indicated) was added to this reaction, and incubated for a further 10 min at 30°C. The reactions were stopped by addition of 20μl of 150mM phosphoric acid, then 50μl of the mixture was

spotted onto P81 phosphocellulose paper and the papers washed and analysed as described above.

Results

Prediction of residues involved in the phosphate binding site.

The three dimensional structure of GSK3 has not yet been elucidated. However, it is possible to model it, based on related kinases whose structures have been solved. The general structure of protein kinases consists of a small lobe, associated primarily with the ATP binding site, and a large lobe that, in PKA, is associated with the peptide substrate binding site. The active site lies in the cleft between the two lobes.

In order to predict residues involved in the putative phosphate binding site, we first took into consideration that many GSK3 substrates require a phosphorylated residue (primed phosphate) four residues C-terminal to the actual phosphorylation site. In the structure of PKA (Fig 1A), the potent peptide inhibitor (PKI) occupies the position of a substrate. If the substrate is positioned similarly in GSK3, then the phosphate binding site should be restricted to residues in the boxed region in Fig 1A. Cyclin-dependent protein kinases (CDKs), to which GSK3 is most closely related, have a similar structure (Fig 1B). The boxed region contains the $\alpha 1$ helix of the small lobe and the residues of the activation loop. We initially focussed our attention on the $\alpha 1$ helix (residues 92-102 of GSK3β) which is compared to the corresponding sequences of CDK2 and PKA in Fig 1C. We assumed that a Lys or Arg residue would be most likely to form part of the putative phosphate binding site and therefore we mutated Arg96 as well as other residues (positively charged residues), to other amino acids. As a control, Leu128 in GSK3β, which we predicted should not be involved in the

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phosphate interaction, was also mutated to Ala. We then compared the ability of the wild-type and mutant enzymes to phosphorylate primed versus non-primed substrates.

5   We first tested the specific activities of the mutant enzymes towards the pGS peptide, which is the most commonly used substrate for measuring GSK3 activity. Its sequence is related to the sites on glycogen synthase phosphorylated by GSK3 (YRRAAVPPS*PSLS*RHSS*PHQSpEDEEE). The phosphorylated serine (Sp) in this peptide, which is phosphorylated by

10   CK2 *in vivo*, acts as the initial primed phosphate triggering the hierarchial phosphorylation of the other serine residues by GSK3 (denoted by asterisks) [13]. The mutation of Arg96 to Ala was found to severely impair its ability to phosphorylate the pGS substrate (Fig 2A), GSK3β [R96A] retaining less than 1% of the activity of the wild-type enzyme. The activity of the

15   GSK3β[LI28A] mutant was similar to that of the wild type enzyme.

The unphosphorylated "non-primed" GS peptide is an extremely poor substrate for GSK3, being phosphorylated about 500-fold more slowly. Nevertheless we were able to accurately and reproducibly measure its

20   phosphorylation by GSK3β (see Methods and inset of Fig 2B). This was catalysed by GSK3, and not by traces of a contaminating kinase, because activity towards either the GS or pGS substrates was suppressed to the same extent by LiCl or Ro 31-8220 (two well established inhibitors of GSK3) in the mutants as in wild-type GSK3. However, in contrast to the

25   phosphorylation of the pGS substrate, the GSK3β[R96A] mutant showed similar activity to the wild type enzyme (and GSK3β[LI28A]) towards the "non-primed" GS peptide (Fig 2B).

We then measured the ability of the enzymes to phosphorylate another "nonprimed" substrate, namely bacterially-expressed Axin [275-510]. The GSK3β[R96A] mutant phosphorylated Axin [275-510] at a similar rate to the wild-type enzyme (Fig 2C), which reproduces the findings obtained with

5   the "non-primed" GS peptide substrate (Fig 2B). However, despite the fact that GSK3β[L128A] retained the specificity of the wildtype enzyme towards primed substrates, the phosphorylation of Axin [275-510] was greatly impaired in this mutant (see Figure 11).

10   We tested whether the activity of these mutants was altered towards primed synthetic phospho-peptides corresponding to the sites on glycogen synthase and eIF2B phosphorylated by GSK3. The mutation of Arg96 to Ala in the small lobe of the kinase domain was found to severely impair the phosphorylation of these peptides, as well as glycogen synthase itself (Fig.

15   8). In contrast, the activity of the Arg96Ala mutant was similar to wild-type GSK3 when assayed against non-primed substrates, such as Axin (Fig. 8). The mutation of several other basic residues in the vicinity of Arg96 (including Arg92Ala and Lys94Ala) did not affect selectivity towards either class of substrate, suggesting that Arg96 interacts directly with the priming

20   phosphate. This is strongly supported by the finding that the replacement of Arg96 by another basic residue (Lys) also abolished activity towards primed substrates, but not non-primed substrates (Fig. 8).

These findings support the existence of a phosphate binding site in GSK3

25   which forms an interaction with the phosphate of primed substrates, and demonstrate that mutation of Arg96 to Ala or Lys disrupts this site. They also suggest that Axin binds to a region of GSK3 distinct from the phosphate-binding site.

Inhibition of GSK-3 activity by a phospho-peptide derived from its N-terminus

The phosphorylation of GSK3β at Ser9, in response to insulin and growth

5   factor signalling, inactivates the kinase [1 8]. However, the mechanism by which this occurs is not understood. We reasoned that the same phosphate binding site occupied by the "primed phosphate" of substrates might also be occupied by the Ser9 residue when it is phosphorylated, thereby competing for substrate binding. To test this hypothesis, we initially investigated

10   whether a phosphopeptide derived from the N-terminus of GSK3β (NT-Ptide or NT-Ptide-11: RPRTTSpFAESC, where Sp is phosphoserine, corresponding to residues 4 to 14 of GSK3β) could inhibit the activity of GSK3β. Indeed, addition of increasing amounts of NT-Ptide inhibited the activity of wild-type GSK3β towards the pGS peptide (Fig 3A and 10A).

15   This effect was very specific, since neither the unphosphorylated N-terminal peptide (NT-tide or NT-tide-11) nor a control phospho-peptide (of the same length) were able to inhibit pGS phosphorylation (Fig 3A and Fig 10A).

This implies that the phospho-serine residue and the particular amino acid

20   sequence surrounding it are both important for inhibition.

We next examined the effect of NT-Ptide (NT-Ptide-11) on the GSK3β mutants. NT-Ptide had no effect on the phosphorylation of pGS by GSK3β[R96A] (Fig 3B), while the GSK3β[L128A] mutant again behaved

25   like the wild-type enzyme. This implies that Arg96 interacts specifically with the phosphorylated N-terminus.

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Thus, in effect, NTptide-11 and the phosphorylated N-terminus of GSK3 exert their inhibitory effects by acting as pseudosubstrates. This was confirmed by the finding that NTptide-11 became a substrate for GSK3 when the proline (equivalent to Pro5 of GSK3β) was changed to serine (RSRTTSpFAESC), phosphorylation occurring at the mutated serine (data not shown).

In vitro, Ser9 phosphorylation ablates the activity of GSK3 towards all substrates studied, including Axin (data not shown). Consistent with this finding, Axin [275-510] phosphorylation by wild-type GSK3β was also inhibited by NT-Ptide (Fig 3C), but not by the non-phosphorylated NT-tide or the control phosphopeptide. In contrast, Axin [275-510] phosphorylation catalysed by GSK3β[R96A] was not inhibited by any of the peptides.

Preincubation with phosphoserine can prevent to a certain extent (but not completely) inhibition by NTPtide-11 of the Axin phosphorylation activity of wild-type GSK3β, ie phosphoserine reduces the inhibition caused by NTPtide-11 (Figure 14). Phosphoserine may compete with NTPtide-11 for binding to the phosphate binding site but may not occupy the catalytic site.

The phosphorylated N-terminal Ser occupies the same site as the phosphate of primed substrates

The data presented above indicated that the same phosphate binding site might be involved in interacting with the phosphoserine residue at the N-terminus of GSK3p, as well as the "primed phosphate". If this were the case, then it would be predicted that the inhibition of GSK3β by PKB would be competed by an excess of the pGS substrate peptide. To test this, GSK3β was phosphorylated at Ser9 and assayed using different concentrations of the pGS peptide. These experiments showed that the inhibition of GSK3β

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by Ser9 phosphorylation is progressively lost as the concentration of the "primed" substrate peptide is increased (Fig 4 and Fig 9B), thereby confirming our hypothesis.

5   *GSK3β [R96A] is resistant to inhibition by PKB.*

Furthermore, we reasoned that if the same residues are involved in interacting with the phosphates of primed substrates and phosphorylated Ser9, then the phosphate binding site mutant should be resistant to inhibition by PKB. In the experiment shown in Fig 5 and Fig 9A, wild-type GSK3β

10   and GSK3β[L128A] were inhibited up to 90% (by preincubation with PKB) at the concentration of pGS substrate used in this experiment. However, GSK3β[R96A] was completely resistant to inhibition, despite the fact that Ser9 was still phosphorylated (to the same extent and at a similar rate to wild-type GSK3; data not shown).

15

Discussion

GSK3 is an unusual protein kinase in two respects. Firstly, it has a unique specificity in requiring a C-terminal "priming" phosphate to catalyse the phosphorylation of several of its key substrates. Secondly, it is one of

20   relatively few protein kinases that are inactivated by phosphorylation. In this paper we demonstrate that these apparently distinct properties are actually connected through the utilisation of a common phosphate binding site. Thus the mutation of a single residue (Arg96), simultaneously abolishes the ability of GSK3β to phosphorylate "primed" substrates (Fig 2

25   and Fig 8) and to be inhibited by the phosphorylation at Ser9 (Fig 5 and Fig 9A). In contrast, the "non-primed" substrates we tested were utilised with equal efficiency by either wild type GSK3β or GSK3β[R96A] (Fig 2 and Fig 8). This suggests that Arg96 may play a key role in interacting with

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both the "primed phosphate" of substrates and phosphorylated Ser9. Thus the unusual specificity requirement of GSK3 may have contributed to the evolution of a novel mechanism for its inactivation. A similar mechanism presumably operates in the GSK3α isoform, inactivation in this case resulting from the interaction of phosphorylated Ser21 with Arg159, the residue equivalent to Arg96 of GSK3β.

Arg96 was selected as one of several basic residues that were potentially capable of interacting with the "primed phosphate" of substrates, based on the assumption that such substrates would bind to GSK3β in a similar manner to the way in which substrates bind to PKA or CDK2 (Fig 1). However, we also mutated two other basic residues in the α1 helix, namely Arg92 and Lys94. Unlike the Arg96Ala mutation, the substitution of Lys94 by Ala did not differentially suppress GSK3β activity towards primed substrates. This did occur when Lys94 was mutated to Glu, but this may be an indirect effect resulting from an electrostatic interaction between Glu94 and Arg96. The Arg92Ala mutation behaved similarly to the wild type enzyme, although it was overall less active towards all substrates tested (data not shown).

Further evidence that the same binding site on GSK3β is used by the "primed phosphate" of substrates and the phosphorylated Ser9 was obtained by the finding that the degree of inhibition resulting from phosphorylation by PKB depended on the substrate concentration. Inhibition decreased progressively as the concentration of the "primed" substrate increased (Fig 4 and Fig 9B). Thus the degree of inhibition of GSK3 *in vivo* towards any physiological substrate will depend on the substrate's affinity for GSK3, its mode of interaction with GSK3 and the local concentration of the substrate.

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It may therefore vary from substrate to substrate and be quite different from the inhibition measured *in vitro* using high concentrations of synthetic peptide substrates. For example, it has been reported that GSK3 activity is only suppressed by about 50% in response to insulin, based on assays using

5   high concentrations of synthetic peptide substrates [18]. It is therefore possible that the extent of inhibition of GSK3 *in vivo* is actually greater than has hitherto been realised.

The residues equivalent to Ser9 and Arg96 of GSK3β are conserved in all

10   vertebrate and invertebrate GSK3 homologues identified to date, suggesting that this mechanism may be universal in animal cells.

Additional evidence that supports the ideas advanced above comes from the finding that a phospho-peptide corresponding to the sequence surrounding

15   Ser9 (NT-Ptide-11) inhibited GSK3β activity towards the "primed" pGS substrate, whereas the non-phosphorylated peptide (NT-tide-11) did not (Fig 3 and Fig 10A). This is not only consistent with our hypothesis, but implies that the phosphorylated N-terminus of GSK3β acts as a pseudosubstrate, with the phosphorylated Ser9 occupying the same position as the "primed

20   phosphate" of substrates. Furthermore, it highlights the fact that the surrounding residues are also important in this respect, since an unrelated phosphopeptide cannot inhibit GSK3β. Only the combination of the phosphoserine in the context of the surrounding residues was able to compete with the primed peptide.

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Indeed, the fact that a serine-substituted version of the phospho-peptide ([P/S]NTptide-11) became a substrate for GSK3 confirms that this is the

case. Furthermore, an N-terminally truncated version of this peptide (NTptide-8) can inhibit the phosphorylation of primed GSK3 substrates in a very specific and selective manner due to the fact that it no longer occupies the catalytic site, and competes for binding of primed substrates to the

5   phosphate-binding site on GSK3 (Fig. 10B). In contrast, NTptide-8 had no effect on the phosphorylation of non-primed substrates. Understanding precisely how this phospho-peptide interacts with GSK3 will be important in the design of selective GSK3 inhibitors that interact with the phosphate-binding site.

10

GSK3 is not only involved in insulin signalling, but also in the Wnt signalling pathway where it controls cell fate during embryonic development (see Introduction). Previous studies conducted in our laboratory demonstrated that Axin, a key component of the Wnt signalling

15   pathway and a substrate of GSK3, forms a complex with GSK3 by interacting at a site distinct from that occupied by glycogen synthase, the pGS peptide or other primed substrates. This was revealed by the finding that a GSK3-binding peptide derived from the human FRAT1 oncogene inhibited the phosphorylation of Axin and βcatenin, by displacing Axin

20   from GSK3 [21]. This peptide did not inhibit the phosphorylation of the "primed" pGS or eIF2B substrate peptides or glycogen synthase purified from muscle, which is already "primed" for phosphorylation by GSK3. Thus the displacement of Axin by FRAT1 allows some functions of GSK3 to proceed, while blocking others.

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The specificity and regulation of GSK3 is governed by different mechanisms in the Wnt and insulin signalling pathways (Fig. 12). The main mechanism leading to inhibition of GSK3 in response to Wnts does not

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involve the phosphorylation of Ser9 (Ruel *et al* (1999) *J. Biol. Chem.* **274**, 21790-21796; Ding *et al* (2000) *J. Biol. Chem.* **275**, 32475-32481), but instead may involve displacement of Axin from its specific binding site on GSK3 as a result of the binding of other proteins, such as GBP/FRAT and 5 Dishevelled (Li *et al* (1999) *EMBO J.* **18**, 4233-4240; Farr *et al* (2000) *J. Cell. Biol.* **148**, 691-702). Since a GSK3-binding peptide derived from FRAT 1 inhibits the phosphorylation of Axin and β-catenin, but does not affect the phosphorylation of primed substrates, such as glycogen synthase or eIF2B (Thomas *et al* (1999) *FEBS Lett.* **458**, 247-251), this may provide 10 a mechanism for restricting the effects of Wnts to the specific subset of GSK3 substrates required to transduce this developmental signal. In contrast, in the insulin signalling pathway, the activity of GSK3 towards primed substrates, such as glycogen synthase or eIF2B, is suppressed by phosphorylation of the N-terminus, through competition for the same 15 phosphate-binding site. The common feature between the effects of Wnts and insulin is that, in each case, the inhibition of GSK3 is intimately linked to the specific requirements of particular substrates for their phosphorylation. Furthermore it now appears that, within the cell, the population of GSK3β bound to Axin does not become phosphorylated at 20 Ser9 in response to insulin (Ding *et al* (2000)). This provides a mechanism for restricting the effects of insulin to the GSK3 substrates that control glycogen and protein synthesis, thereby preventing insulin from inhibiting the GSK3-catalysed phosphorylation of components of the Wnt signalling network.

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In the present study, the distinct interaction of Axin with GSK3, compared to other substrates, was confirmed by mutational analysis. Thus the phosphorylation of Axin [275-510] was not impaired in GSK3β[R96A]

(Figs 2C and 2D) which was inactive towards the pGS peptide substrate (Fig 2A). Conversely, GSK3β[L128A] behaved similarly to wild-type GSK3β with respect to pGS phosphorylation, but was greatly impaired in its ability to phosphorylate Axin [275-510]. This implies that Axin and pGS have

5   independent binding sites on GSK3 and that Leu128 may participate in the binding of Axin. In this regard, it is of interest that Leu128 is equivalent to Leu155 of 3 -phosphoinositide-dependent protein kinase- 1 (PDK 1), which is located in a hydrophobic pocket that is critical for substrate recognition by this protein kinase [23].

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The specificity of the protein kinase CK2 shows some resemblance to that of GSK3 in that a cluster of acidic residues immediately C-terminal to the phosphorylation site, and especially an acidic residue at the n+3 position, are critical for specific substrate recognition. Moreover, phosphoserine and

15   phosphotyrosine can substitute for the acidic residues [24]. Interestingly, these acidic residues interact with many basic residues in CK2, including a cluster of eight located between residues 74 and 83 in the α1 helix [25], where Lys77 is equivalent to Arg96 of GSK3β. This is consistent with Arg96 playing a direct role in phosphate binding by GSK3β.

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Although Axin is a "non-primed" substrate that does not utilise the phosphate binding site, we show for the first time that phosphorylation of Ser9 abolishes the activity of GSK3β towards Axin [275-510] and β-catenin *in vitro*. This is not surprising if the phosphorylated N-terminus is acting as

25   a pseudosubstrate, because access of all substrates to the active site will be blocked. However, whether this occurs *in vivo* with full length Axin that is present in a multiprotein complex with GSK3 and other proteins, in Wnt-responsive cells is unknown. This is clearly a critical question to resolve, in

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order to evaluate whether phosphorylation of Ser9 could be a mechanism for integrating or synergising the effects of Wnts with those of agonists that activate phosphatidylinositol 3-kinase and PKB (Fig 6).

5   Since insulin inhibits GSK3, cell permeant drugs that inhibit this protein kinase may mimic some of the actions of this hormone and have therapeutic potential for the treatment of NIDDM. However, such drugs might have serious side effects if they also prevented the phosphorylation of Axin and β-catenin leading to the stabilisation of β-catenin and stimulation of

10   oncogenic gene transcription. The overexpression of non-phosphorylatable (and hence non-degradable) mutants of β-catenin is known to be oncogenic, and such mutants have been found in malignant melanomas and other cancers [9] [10]. The present work may therefore be significant because it suggests approaches to the design of novel GSK3 inhibitors that, by binding

15   to the phosphate binding site instead of the catalytic centre, would prevent the phosphorylation of metabolic proteins relevant to insulin signalling without affecting the phosphorylation of Axin and β-catenin. Indeed one of the goals for the development of drugs that target protein kinases must be to develop inhibitors which suppress the phosphorylation of different

20   substrates, thereby allowing the selective inhibition of one function and not another.

Relatively specific cell permeant inhibitors of GSK3 have recently been described that, in cell based assays, mimic the ability of insulin to stimulate

25   the conversion of glucose to glycogen (Coghlan *et al* (2000) *Chem. Biol.* 7, 793-803). However, since these compounds are ATP-competitive, they inhibit the phosphorylation of every GSK3 substrate tested to a similar extent. For this reason, they also stimulate β-catenin-dependent gene

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transcription (Coghlan *et al* (2000)) and therefore have the potential to be oncogenic. The identification of a specific phosphate-binding site in GSK3 has opened up a new opportunity to develop drugs that selectively inhibit the phosphorylation of key proteins involved in mediating the metabolic actions of insulin that are suitable for the long term treatment of diabetes, without the potential to be oncogenic. They may also be suitable for the treatment of other diseases in which GSK3 has been implicated (Hetman *et al* (2000) Role of glycogen synthase kinase-3beta in neuronal apoptosis induced by trophic withdrawal. *J. Neurosci.* **20**, 2567-2574; Lovestone *et al* (1994) Alzheimer's disease-like phosphorylation of the microtubule-associated protein tau by glycogen synthase kinase-3 in transfected mammalian cells. *Curr. Biol.* **4**, 1077-1086; Sperber *et al* (1995) Glycogen synthase kinase-3 beta phosphorylates tau protein at multiple sites in intact cells. *Neurosci. Lett.* **197**, 149-153).

Further data strengthens the view that the N-terminus is a naturally occurring pseudosubstrate. A modified form of NT-Ptide that has a Ser at position 17 of GSK3α instead of the Ala normally present in the N-terminus, is a very good substrate of GSK3, probably equivalent to other primed peptide substrates. Thus it is likely that the native N-terminus (with Ala (in GSK3α) or Pro (in GSK3β) is a naturally occurring pseudosubstrate when Ser 9 or Ser21 is phosphorylated.

Results obtained on testing several other substrates, including tau, glycogen synthase protein and peptides (primed and non-primed) derived from eIF2B are consistent with the results and explanations given earlier in Example 1. Similar results may be obtained with β-catenin and tau (when it has been primed).

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Example 2: screening methods

Fluorescence Resonance Energy Transfer (FRET)-based methods

It is quite clear that FRET can be obtained between chromophores, for example fluorophores, for example appropriate Green Fluorescence Proteins (GFPs) fused to interacting proteins. In an extension of this, this methodology could also be used to look for interactions between different parts of the same protein. FRET measurements may be more likely to be useful if the positioning of the fluorophores, for example GFPs is such that they are expected to be in close proximity, for example based on structural information. From our model that indicates that Ser9-P interacts with Arg96, we can now determine appropriate positions to locate two fluorophores, for example two GFPs so that they would be at close proximity when Ser9 is phosphorylated but not when Ser9 is not phosphorylated. This could lead to a GFP1-GSK3-GFP2 ([fluorophore]-[C-terminal truncated GSK3]–fluorophore] construct or [NT-Ptide]-[GFP] and [GSK3-GFP2] constructs that could be used to screen for compounds that release the interaction of the N-terminal phosphorylated sequence with the phosphate binding site. Positive compounds may then be re-screened to determine whether they interact only with the phosphate binding site (hence inhibiting only the phosphorylation of primed substrates) or whether they inhibit completely the enzymatic activity. Both types of compounds could

be useful. With some types of fluorophores such as GFPs, the GFP1-GSK3-GFP2 could also be produced in cells and screens could be performed *in vivo* for compounds that modify the FRET.

5   The GSK3 structure may be modelled based on CDK2 structures when considering where to position fluorophores. It should be noted that GFPs are almost as big as the whole GSK3. The C-terminal residues of GSK3 cannot be modelled in this way since they lack homology with CDK2. We predict that the phosphorylation of Ser9 in GSK3β would move the N-terminus to a

10   position where FRET would be detectable between appropriate GFPs. Lack of phosphorylation would lead to a structure where the active site should be available for interaction with substrates, hence the N-terminus should have moved considerably. FRET can detect small changes in the distance between the corresponding fluorophores. The efficiency of FRET decreases

15   as a function of $Ef=1/d^6$. Therefore small changes in the distance between the fluorophores is transduced to big differences in FRET.

Phage Display methods

Phage display could be a possible methodology of choice in order to obtain

20   a compound that would inhibit the phosphorylation of primed substrates without affecting the phosphorylation of substrates that do not require a priming phosphate. Polypeptides that interact with wtGSK3 (and certain mutants of GSK3) but do not interact with the 96A mutant would be selected. These compounds may be able to differentially inhibit the

25   phosphorylation of substrates. These polypeptides could be used for crystallography studies or other structure determination/prediction methods, and hence serve as models for drug design.

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Other methods

Park & Raines (2000) *Nature Biotechnol* **18**, 847-851 "Genetic selection for dissociative inhibitors of designated protein-protein interactions" describes methods for searching for polypeptides that would disrupt protein-protein

5   interactions. Such methods may be useful in relation to the present invention. However, the methods may require identification of a non-phosphorylated polypeptide that binds to the phosphate binding site. In addition, the method is based on the expression of the protein partners in *E.coli*, which may not lead to a well folded GSK3. Thus, other methods may

10   be preferable.

Fusion proteins comprising polypeptides that can interact with the phosphate binding site.

A nucleotide coding for a fusion polypeptide comprising a specific

15   polypeptide inhibitor may be useful (for example in detecting the polypeptide and its interactions). For example, the sequence of a specific peptide that can interact with the phosphate binding site (eg. obtained from phage display analysis) may be targeted to specific cells and be expressed as a fusion protein, for example as a GFP or GST fusion protein or within

20   loops of proteins such as thioredoxin, for which the structure is known and there is experience in the literature (Curr Biol 1997 Nov 1;7(11):860-9).

Example 3: Screening of GSK3 phosphate binding site mutations in humans

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It is possible that such mutations occur in humans and could lead to a predisposition to disorders of glucose storage and metabolism. A disorder may occur where glucose levels are low under circumstances where glycogen is present, and cleaved upon the correct stimulus, but the produced

glucose is then converted back to glycogen. This may be caused as follows: insulin stimulates glycogen synthase activity (through inhibition of GSK3 and dephosphorylation of glycogen synthase (GS)). People who carried a germline mutation in GSK3, for example GSK3β[R96A], would not be

5　　expected to respond correctly to insulin because their GSK3 would not be inhibited by Ser9 phosphorylation, so they would not be able to activate GS in response to insulin. These people may be insulin-resistant diabetics. Furthermore, in "normal individuals" in the absence of insulin (resting state) wild-type GSK3 is active. GS is phosphorylated under these conditions, and

10　　therefore inactive, thus glycogen is not stored. But those individuals carrying a mutation in GSK3 which disrupted the phosphate binding site, would not be able to phosphorylate glycogen synthase efficiently, thus it would remain active, and convert all available glucose to glycogen. Thus such germline mutations in GSK3 may predispose to metabolic disorders

15　　and screening could be carried out to identify these individuals.

Example 4: NTPtide-11/Axin based kinase assay screen for identifying compounds that bind to the phosphate binding site of GSK3.

The ability of GSK3 to phosphorylate Axin may be determined using a

20　　kinase assay as described in the legend to Figure 14. The data shown in Figure 14 shows that 10 mM phosphoserine can reduce the inhibition by NTPtide-11 of the activity of GSK3 towards Axin to approximately 76% of the control, presumably by binding to the phosphate binding site. Similar effects are observed at lower concentrations of phosphoserine (data not

25　　shown). This screening method or variations thereof may be used to identify compounds interacting with the phosphate binding site on GSK3.

CLAIMS

1. A method of identifying a compound that modulates the protein kinase activity of GSK3, comprising the steps of (1) determining the effect of a test compound on the protein kinase activity of GSK3 and/or a mutant thereof, and (2) selecting a compound capable of inhibiting the protein kinase activity of GSK3 towards (i) a phosphate-dependent (primed) substrate and (ii) a non-phosphate dependent substrate to different extents, wherein when the effect of a test compound is determined on the protein kinase activity of native GSK3 or a fusion thereof but not on a mutant of GSK3, a compound is selected that is capable of inhibiting the protein kinase activity of GSK3 towards a phosphate-dependent (primed) substrate to a greater extent than towards a non-phosphate dependent substrate.

2. A method of identifying a compound that modulates the protein kinase activity of GSK3, comprising the step of determining the effect of a test compound on the protein kinase activity of inhibited GSK3.

3. The method of claim 2 comprising the step of determining the effect of the test compound on the protein kinase activity of inhibited GSK3 towards a non-phosphate dependent substrate.

4. The method of claim 3 wherein a compound that increases the protein kinase activity of inhibited GSK3 towards the non-phosphate dependent substrate is selected.

5. The method of claim 1, 3 or 4 wherein the non-phosphate dependent substrate comprises the sequence S/T-$(X)_n$-Z (wherein n is at least 3,

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preferably 3, 4, 5 or 6 or up to 14, most preferably 3, and preferably less than about 20, 30 or 40) and is phosphorylated on the S/T residue by GSK3, wherein Z is not phosphoserine or phosphothreonine (non-primed substrate) and wherein a polypeptide identical to the non-primed substrate with the

5 exception that Z is replaced by a phosphoserine or phosphothreonine residue ("primed" or phosphate-dependent substrate) is a better substrate for phosphorylation on the S/T residue by GSK3 than the non-primed substrate.

6. The method of claim 1, 3 or 4 wherein the non-phosphate dependent
10 substrate is axin, β-catenin, tau, c-myc, myb or c-jun or suitable variant, fragment, derivative or fusion thereof.

7. The method of any of the preceding claims wherein the inhibited GSK3
15 is GSK3 phosphorylated at the residue equivalent to Ser 9 of full-length human GSK3β.

8. The method of any of claims 1 to 6 wherein the inhibited GSK3 is GSK3 bound to a compound or polypeptide that interacts with a phosphate binding
20 site of the GSK3 that is defined by residues including arginine 96 of full-length GSK3β, and/or comprising the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, wherein the polypeptide is not a substrate of GSK3.

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9. The method of claim 8 wherein the polypeptide has an amino acid sequence derivable from a phosphate-dependent (primed) substrate of GSK3 in which the serine or threonine residue that is phosphorylatable by GSK3 is replaced by a non-phosphorylatable residue.

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10. A method of identifying a compound that modulates the protein kinase activity of GSK3, wherein the effect of the said compound on the rate or degree of phosphorylation of a substrate polypeptide of GSK3 by GSK3 in

5   the presence of an interacting compound or polypeptide is determined, and a compound that modulates the said rate or degree of phosphorylation is selected, wherein the interacting compound or polypeptide interacts with the phosphate binding site of the GSK3 that is defined by residues including arginine 96 of full-length GSK3β and/or comprises the amino acid

10   sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, wherein the polypeptide is not a substrate of GSK3.

11. A method of identifying a compound that modulates the protein kinase

15   activity of GSK3, comprising the step of determining the effect of the compound on the phosphorylation by GSK3 of a non-primed polypeptide, wherein the non-primed polypeptide comprises the sequence $S/T-(X)_n-Z$ (wherein n is at least 3, preferably 3, 4, 5 or 6 or up to 14, most preferably 3, and preferably less than about 20, 30 or 40) and is phosphorylated on the

20   S/T residue by GSK3, wherein Z is not phosphoserine or phosphothreonine and wherein a polypeptide identical to the non-primed polypeptide with the exception that Z is replaced by a phosphoserine or phosphothreonine residue (primed substrate) is a better substrate for phosphorylation on the S/T residue by GSK3 than the non-primed substrate.

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12. A method of identifying a compound that modulates the protein kinase activity of GSK3, comprising the step of determining the effect of the compound on the protein kinase activity of, or ability of the compound to bind to, (1) GSK3 mutated at a residue defining at least part of the

phosphate binding site of GSK3 that is defined by residues including arginine 96 of full-length GSK3β, for example the residue equivalent to arginine 96, and/or the residue equivalent to lysine 94, of full-length human GSK3β and/or (2) GSK3 mutated at a residue defining at least part of the

5   GSK3 site, alteration of which modulates activity of GSK3 towards non-phosphorylated substrates, that is defined by residues including leucine 128 of full-length human GSK3β, for example the residue equivalent to leucine 128 of full-length human GSK3β.

10 13. A method according to claim 12 further comprising determining the effect of the compound on the protein kinase activity of, or ability of the compound to bind to, GSK3 which is not mutated at the said residue defining at least part of the phosphate binding site of GSK3 that is defined by residues including arginine 96 of full-length GSK3β, for example the

15 residue equivalent to arginine 96, and/or the residue equivalent to lysine 94, of full-length human GSK3β and/or (2) defining at least part of the GSK3 site, alteration of which modulates activity of GSK3 towards non-phosphorylated substrates, that is defined by residues including leucine 128 of full-length human GSK3β, for example the residue equivalent to leucine

20 128 of full-length human GSK3β.

14. The method of any one of claims 2, 12 or 13 wherein the effect of the compound on the rate or degree of phosphorylation of a phosphate-dependent (primed) substrate, for example glycogen synthase or eIF2B or

25 suitable variant, fragment, derivative or fusion thereof, is determined.

15. The method of any one of claims 2, 12 or 13 wherein the effect of the compound on the rate or degree of phosphorylation of a non-phosphate-

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dependent substrate, for example axin or β-catenin or suitable variant, fragment, derivative or fusion thereof, is determined.

16. The method of any one of claims 2 to 15 claims wherein a compound is selected that decreases the protein kinase activity of GSK3 towards a primed substrate and does not affect or increases the protein kinase activity of GSK3 towards a non-phosphate-dependent substrate.

17. A method of identifying a compound that modulates the protein kinase activity of GSK3, wherein the ability of the compound to inhibit, promote or mimic the interaction of GSK3 with an interacting compound or polypeptide is measured and a compound that inhibits, promotes or mimics the said interaction is selected, wherein the interacting compound or polypeptide interacts with the phosphate binding site of the GSK3 that is defined by residues including arginine 96 of full-length GSK3β and/or comprises the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, wherein the polypeptide is not a substrate of GSK3.

18. The method of claim 17 wherein the interaction of GSK3 with an interacting compound or polypeptide is measured using fluorescence resonance energy transfer (FRET).

19. The method of claim 17 wherein the interaction of GSK3 with an interacting compound or polypeptide is measured using surface plasmon resonance.

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20. The method of claim 17, 18 or 19 wherein the interacting polypeptide interacts with a phosphate binding site that is defined by residues including arginine 96 of full-length GSK3β.

5   21.   The method of any of the previous claims wherein the interacting polypeptide is part of the GSK3 polypeptide chain.

22.   The method of any one of claims 17 to 21 wherein the interaction is an intramolecular interaction.

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23.   The method of any of the preceding claims wherein the GSK3 is mammalian.

24.   The method of any of claims 1 to 22 wherein the GSK3 is derivable

15   from a parasitic or pathogenic or potentially pathogenic organism.

25.   A method of selecting or designing a compound that modulates the activity of GSK3, the method comprising the step of using molecular modelling means to select or design a compound that is predicted to interact

20   with GSK3, wherein a three-dimensional structure of the phosphate binding site of the GSK3 that is defined by residues including arginine 96 of full-length GSK3β and/or with a three-dimensional structure of an interacting polypeptide, for example comprising the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-Ptide or

25   NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, is compared with a three-dimensional structure of a compound, and a compound that is predicted to interact with the said phosphate binding site is selected.

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26. A method of selecting or designing a compound that modulates the activity of GSK3, the method comprising the step of using molecular modelling means to select or design a compound that is predicted to interact with GSK3, wherein a three-dimensional structure of an interacting

5   polypeptide comprising the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, is compared with a three-dimensional structure of a compound, and a compound that is predicted to interact with the active site is selected.

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27. A method of selecting or designing a compound that modulates the activity of GSK3, the method comprising the step of using molecular modelling means to select or design a compound that is predicted to interact with GSK3, wherein a three-dimensional structure of the GSK3 site,

15   alteration of which modulates activity of GSK3 towards non-phosphorylated substrates, that is defined by residues including leucine 128 of full-length human GSK3β, is compared with a three-dimensional structure of a compound, and a compound that is predicted to interact with the said substrate binding site is selected.

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28. A compound capable of modulating the protein kinase activity of GSK3, wherein the compound inhibits the interaction of GSK3 with an interacting polypeptide, wherein the interacting polypeptide interacts with the phosphate binding site of GSK3 that is defined by residues including

25   arginine 96 of full-length GSK3β and/or comprises the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E, for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof, wherein the compound is not a substrate of GSK3 or a pGS-derivable pseudosubstrate of GSK3.

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29. A compound capable of modulating the protein kinase activity of GSK3, wherein the compound is capable of interacting with the phosphate binding site of GSK3 that is defined by residues including arginine 96 of

5   full-length GSK3β wherein the compound is not a substrate of GSK3 or a pGS-derivable pseudosubstrate of GSK3.

30. A compound capable of modulating the protein kinase activity of GSK3, wherein the compound modulates the rate or degree of

10 phosphorylation of a substrate polypeptide of GSK3 by GSK3 in the presence of an interacting polypeptide, wherein the interacting polypeptide comprises the amino acid sequence RPRTTSpFAESC (NT-Ptide) or TTSpFAESC (NT-Ptide-8) or variant thereof, wherein the compound is not a substrate of GSK3 or a pGS-derivable pseudosubstrate of GSK3.

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31. A compound capable of inhibiting the protein kinase activity of GSK3 wherein the compound inhibits to a greater degree the rate or degree of phosphorylation by GSK3 of (1) a primed substrate polypeptide of GSK3 than (2) a non-phosphate-dependent substrate of GSK3.

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32. A compound identifiable by the method of any one of claims 1 to 27 provided that the compound is not a substrate of GSK3 or a pGS-derivable pseudosubstrate of GSK3.

25 33. The compound of any one of claims 28 to 32 wherein the compound is not a polypeptide.

34. A pharmaceutical composition comprising a compound according to any one of claims 28 to 33 and a pharmaceutically acceptable excipient.

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35. A compound according to any one of claims 28 to 33 or composition according to claim 34 for use in medicine.

36. A mutated GSK3, wherein one or more residues defining the phosphate binding site of GSK3 that is defined by residues including arginine 96 of full-length human GSK3β is mutated.

37. A mutated GSK3, wherein one or more residues defining the GSK3 site, alteration of which modulates activity of GSK3 towards non-phosphorylated substrates, that is defined by residues including leucine 128 of full-length human GSK3β is mutated.

38. The mutated GSK3 of claim 36 wherein a mutated residue(s) is the residue equivalent to residue arginine 96 of full-length human GSK3β.

39. The mutated GSK3 of claim 37 wherein a mutated residue(s) is the residue equivalent to residue leucine 128 of full-length human GSK3β.

40. The mutated protein kinase of claim 38 or 39 wherein the residue at the position equivalent to residue Arg 96 is mutated to an Ala or Lys, or the residue at the position equivalent to residue Leu 128 of full length human GSK3β is mutated to an Ala.

41. A preparation comprising GSK3, and a second, interacting compound, wherein the interacting compound is capable of interacting with the phosphate binding site of GSK3 that is defined by residues including arginine 96 of full-length GSK3β and/or comprises the amino acid sequence A/P-R-T-S/T-Sp-F-A-E or T-S/T-Sp-F-A-E for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or variant thereof.

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42.   The preparation of claim 41 wherein the said preparation further comprises a substrate polypeptide of GSK3.

43.   A method of modulating the protein kinase activity of GSK3 wherein the GSK3 is exposed to a compound or composition as defined in any one of claims 28 to 34.

44.   The method of claim 43 wherein the compound or composition inhibits to a greater degree the rate or degree of phosphorylation by GSK3 of (1) a primed substrate polypeptide of GSK3 than (2) a non-phosphate-dependent substrate of GSK3.

45.   The method of claim 43 wherein the compound or composition inhibits to a greater degree the rate or degree of phosphorylation by GSK3 of (1) a non-phosphate-dependent substrate of GSK3 than (2) a primed substrate polypeptide of GSK3.

46.   A method of modulating in a cell the protein kinase activity of GSK3, wherein a recombinant interacting polypeptide is expressed in the cell, wherein the interacting polypeptide interacts with the phosphate binding site of GSK3 but does not prevent phosphorylation of non-phosphate-dependent substrates of GSK3.

47.   An interacting polypeptide as defined in claim 41 or 46.

48.   A polynucleotide encoding a polypeptide according to claim 47 or a mutated protein kinase according to any of claims 36 to 40.

49. A recombinant polynucleotide suitable for expressing a polypeptide according to claim 47 or a mutated protein kinase according to any of claims 36 to 40.

5   50. A host cell comprising a polynucleotide according to claim 48 or 49.

51. A method of making a polypeptide according to claim 47 or a mutated protein kinase according to any of claims 36 to 40, the method comprising culturing a host cell according to claim 50 which expresses said polypeptide

10   or mutated protein kinase and isolating said polypeptide or mutated protein kinase.

52. A polypeptide or mutated protein kinase obtainable by the method of claim 51.

15

53. A cell containing a recombinant nucleic acid suitable for expressing GSK3 and a recombinant nucleic acid suitable for expressing an interacting polypeptide as defined in claim 41 or 46.

20   54. An antibody that with interacts with the phosphate binding site of GSK3 that is defined by residues including arginine 96 of full-length GSK3β or with the site of GSK3, alteration of which modulates activity of GSK3 towards non-phosphorylated substrates, that is defined by residues including leucine 128 of full-length human GSK3β.

25

55. A kit of parts useful in carrying out a method according to any one of claims 1 to 27, comprising at least two of (1) GSK3 which is not a mutated GSK3 according to any one of claims 36 to 40, (2) a mutated GSK3 according to any one of claims 36 to 40 and/or (3) a separate interacting

polypeptide wherein the interacting polypeptide interacts with the phosphate binding site of GKS3 and/or comprises the amino acid sequence NTP-tide, and is not a substrate of GSK3.

5   56. The kit of claim 55 further comprising a phosphate-dependent substrate and/or a non-phosphate-dependent substrate.

57. A polypeptide according to any of claims 47 or 52 or mutated protein kinase according to any one of claims 36 to 40 or polynucleotide according

10   to claim 48 or 49 for use in medicine.

58. A pharmaceutical composition comprising a polypeptide according to any of claims 47 or 52 or mutated protein kinase according to any one of claims 36 to 40 or polynucleotide according to claim 48 or 49 and a

15   pharmaceutically acceptable excipient.

59. The use of a compound or composition or polypeptide or polynucleotide as defined in claim 35 or 58 in the manufacture of a medicament for the treatment of a patient in need of modulation of signalling by GSK3.

20

60. The use of claim 59 wherein the patient is in need of inhibition of phosphorylation of primed substrates of GSK3 but not in need of inhibition of phosphorylation of non-phosphate-dependent substrates of GSK3.

25   61. The use according to claim 59 or 60 wherein the compound or composition inhibits to a greater degree the rate or degree of phosphorylation by GSK3 of (1) a primed substrate polypeptide of GSK3 than (2) a non-phosphate-dependent or non-primed substrate of GSK3.

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62. The use of any of claims 59 to 61 wherein the patient has diabetes.

63. An interacting polypeptide which interacts with the phosphate binding site of GSK3 and/or comprises the amino acid sequence A/P-R-T-S/T-Sp-F-

5   A-E or T-S/T-Sp-F-A-E for example RPRTTSpFAESC (NT-Ptide or NT-Ptide-11) or TTSpFAESC (NT-Ptide-8) or a pseudosubstrate of GSK3, immobilised on a surface of an article suitable for use as a test surface in a surface plasmon resonance method.

10 64. A variant of a polypeptide as defined in claim 63 wherein the phosphoserine or equivalent residue that interacts with the phosphate binding site of GSK3 is replaced by a residue other than phosphoserine, phosphothreonine or an acidic residue.

15 65. A variant as defined in claim 64 immobilised on a surface of an article suitable for use as a test surface in a surface plasmon resonance method.

66. A mutated GSK3 as defined in any one of claims 36 to 40 immobilised on a surface of an article suitable for use as a test surface in a surface

20 plasmon resonance method.

67. A polypeptide consisting of the amino acid sequence $TTSpFAESC(X)_m$ or $RPRTTSpFAESC(X)_m$ wherein m is between 0 and 10, 20, 30, 40, 50, 80, 100, 200 or 500.

25

Figure 1

Figure 2 (page 1 of 2)

◼ **Control**
▨ **+ LiCl (50mM)**
▩ **+ RO 31-8220 (100nM)**

A

pGS peptide

415U/mg

GSK3 activity (% of wild-type)

WT   R96A   L128A

B

GS peptide

autoradiograph
— GSK3
— GS peptide-P

1.33U/mg

GSK3 activity (% of wild-type)

WT   R96A   L128A

Figure 2 (page 2 of 2)

Figure 3 (page 1 of 2)

Figure 3 (page 2 of 2)

Figure 4

Inhibition of GSK3 activity (%) vs pGS peptide (µM)

Figure 5

Figure 6

Wnt   **Insulin, GFs**

N   N

Axin

Glycogen synthase

GSK3   GSK3

FRAT

N

N

Axin   GSK3   GSK3 (Ser9-P)   Glycogen synthase

Cellular response A   Cellular response B   Cellular response C

Figure 7

GSK3β Model
GFP1 shown fused at residue Gly 3

GFP2 shown fused at Pro 346

Figure 8

Figure 9

A

B

A

Figure 10

B

Figure 11

Figure 12

Axin/FRAT
binding site

Phosphate-
binding site

GSK3

small
lobe

large
lobe

N-terminal tail

Wnt

Insulin

FRAT

PKB

inactivated

activated

Key:

GSK3-Axin
complex

FRAT

Phosphorylation site

β-catenin

Glycogen
synthase

Figure 13

Figure 14

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(54) Title: PROTEIN KINASE REGULATION

(57) Abstract: Methods are provided for identifying compounds that are capable of, for example, inhibiting the activity of GSK3 towards phosphate-dependent (primed) substrates to a greater extent than towards non-phosphate-dependent substrates. Mutant GSK3s and other novel polypeptides, polynucleotides and recombinant cells that are useful in such methods are provided. For example, polypeptides useful in modulating the activity of GSK3 are also provided.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   C12Q1/48   C12N9/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   C12Q   G01N   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, EPO-Internal, SCISEARCH, CHEM ABS Data, SEQUENCE SEARCH, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 41854 A (UNIV PENNSYLVANIA ;HARVARD COLLEGE (US)) 13 November 1997 (1997-11-13) the whole document ───	
A	US 6 057 286 A (RING DAVID B ET AL) 2 May 2000 (2000-05-02) the whole document ─── -/──	

[X]	Further documents are listed in the continuation of box C.	[X]	Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	THOMAS G M ET AL: "A GSK3-binding peptide from FRAT1 selectively inhibits the GSK3-catalysed phosphorylation of Axin and beta-catenin" FEBS LETTERS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 458, no. 2, 17 September 1999 (1999-09-17), pages 247-251, XP004260269 ISSN: 0014-5793 cited in the application the whole document	
A	WO 97 22360 A (MEDICAL RES COUNCIL ;UNIV DUNDEE (GB); ALESSI DARIO (GB); COHEN PH) 26 June 1997 (1997-06-26) claim 31; figure 4	
T	FRAME SHEELAGH ET AL: "A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation." MOLECULAR CELL, vol. 7, no. 6, June 2001 (2001-06), pages 1321-1327, XP002193599 ISSN: 1097-2765 the whole document	
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FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.:   28-67

Present claims 28-67 relate to a compound/mutant defined by reference to
a desirable characteristic or property, namely modulating the protein
kinase activity of GSK3, interacting with the phosphate binding site of
GSK3 or being a mutated GSK3 wherein one or more residues defining the
phosphate binding site of GSK3 is mutated.

The claims cover all compounds/mutants having this characteristic or
property, whereas the application provides support within the meaning of
Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for
only a very limited number of such compounds/mutants. In the present
case, the claims so lack support, and the application so lacks
disclosure, that a meaningful search over the whole of the claimed scope
is impossible. Independent of the above reasoning, the claims also lack
clarity (Article 6 PCT). An attempt is made to define the
compounds/mutants by reference to a result to be achieved. Again, this
lack of clarity in the present case is such as to render a meaningful
search over the whole of the claimed scope impossible. Consequently, the
search has been carried out for those parts of the claims which appear to
be clear, supported and disclosed, namely those parts relating to the
NT-Ptides 'see description at page 66!, and the mutants disclosed in the
description pages 69-81.

The applicant's attention is drawn to the fact that claims, or parts of
claims, relating to inventions in respect of which no international
search report has been established need not be the subject of an
international preliminary examination (Rule 66.1(e) PCT). The applicant
is advised that the EPO policy when acting as an International
Preliminary Examining Authority is normally not to carry out a
preliminary examination on matter which has not been searched. This is
the case irrespective of whether or not the claims are amended following
receipt of the search report or during any Chapter II procedure.

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